

New-style abuse of press freedom

A British newspaper, *The Sunday Times*, has so consistently misrepresented the role of HIV in the causation of AIDS that *Nature* plans to monitor its future treatment of this issue, if only to save readers the trouble of buying it.

It is a principle of the open society that newspapers should be free from censorship. Even wrong-headed newspapers deserve that licence. If it were not so, the communities in which they circulate would be impoverished. In the United States, the First Amendment of the Constitution guarantees the right of free expression, but even in countries such as Britain, there is a kind of *de facto* recognition of the principle. Censorship, the enemy of liberty, is abhorrent.

The application of these principles is naturally difficult. Newspapers, like ordinary people and other corporate entities, must obey whatever laws apply (on matters such as sedition) in the regions in which they circulate. Worse, freedom of the press allows newspapers lacking admirable qualities to flourish. In Britain, for example, still free from most constraints on publication, some newspapers value royal tittle-tattle above current affairs, while others flaunt prejudice, political or xenophobic. The great and the good shake their heads over these excesses, muttering that they should be "curbed". (It cannot be long before a privacy bill goes through the House of Commons.) This journal, on the other hand, shares the view that bad newspapers are part of the cost of ensuring that some should be good, but this is hardly a live issue in the reporting of science, where earnestness is more common than obdurate exaggeration. Except, that is, for *The Sunday Times* and for its line on AIDS.

Mystification

That is a mystifying business. For more than two years the most profitable newspaper in Britain has supported the view that HIV is irrelevant to the causation of AIDS. This opinion can be traced to the journalistically proper reporting of Dr Peter Duesberg's well-known dissent from the general opinion that AIDS follows HIV-infection, but *The Sunday Times* has since made the cause its own. The "HIV-hypothesis" is called a "myth". The prime mover in what the newspaper has now dignified as a "campaign" is the journalist Neville Hodgkinson, the newspaper's science editor. In an article in *The Sunday Times* two weeks ago, he likened this "campaign" to the same newspaper's courageous advocacy two decades ago of the argument that the drug thalidomide had caused numerous neonatal malformations when used in the treatment of pre-eclampsia in pregnancy. The claim is laughably irrelevant: the circumstances are very different. And the newspaper was then run by different people under different ownership.

Hodgkinson's most recent article (on 28 November) was

stimulated by an attack on Duesberg's position, under the title "What does this man know that we do not?", appearing a week earlier in the magazine section of the rival *The Sunday Telegraph*. Although meant to "set straight the half-truths and fictions surrounding the disease", Hodgkinson's response is a convenient rehearsal of the newspaper's position. Thus it alleges "self-censorship by ... most newspapers and ... almost all medical and scientific journals" to suppress news of the Duesberg controversy. It goes on to explain that "It was thought during the 1980s that ... millions of lives would be at risk unless the sexually active public changed their ways...", but that "as the years passed without any significant spread of AIDS among heterosexuals, ...this newspaper began to break the consensus."

Caveat

But *The Sunday Times* now insists that it does not accept the whole of Duesberg's view on AIDS, and particularly his assertion that drug-taking (and some medications) may be the true cause; rather, it says, there is "a spectrum of ideas about HIV and its putative role in AIDS" and that "the world of science may have made a terrible blunder, costly in both money and lives, in jumping to the conclusion that in HIV it had found the answer to AIDS".

In passing, *The Sunday Times* singles out this journal for its supposed censorship of Duesberg's "challenge" to orthodoxy on the grounds that it has declined to publish a letter pleading for a reappraisal of the evidence by a "suitable independent group". The innocent-sounding letter was organized by Dr Charles A. Thomas of the Helicon Foundation in San Diego, California, and originally carried 50 signatures; now (according to Hodgkinson) there are 300. But so what? The individuals who are members of the enlarged group command even more varied degrees of respect for judgement on scientific matters than the original signatories. In any case, this journal avoids whenever possible the publication of round-robin letters, whose method is usually hortatory rather than reasonable. It is not, after all, as if readers of this journal have not been fully informed of Duesberg's heterodoxy.

The business of this unpublished letter is nevertheless a helpful pointer to the error of *The Sunday Times*, as of the signatories of the letter. The error is essentially epistemological. At its lowest, it is based on the supposition that the "true" hypothesis can be decided by some kind of democratic process in which people vote their beliefs. Otherwise,



the assumption is, truth is decided by an adversary process in which a logic-chopping "challenge" can see off a favoured hypothesis. In that spirit one might say that cigarette smoking cannot be a cause of lung cancer because some people smoke all their lives and then die of something else. Or that the Sun's energy cannot derive from thermonuclear reactions in the core because the neutrinos it emits are only a third of those predicted by the hypothesis. Popper's advocacy of the falsifiable hypothesis notwithstanding, discordant observations are in the first instance a spur to reconciliation with the most plausible hypothesis, not its rejection.

Disappointment

None of this should conceal the truth that the causation of AIDS has been a profound disappointment to the research community. Almost ten years have passed since HIV was first recognized, but the evidence that it causes AIDS is still epidemiological. The mechanism of the pathogenesis of the disease has not yet been uncovered (although parts of Duesberg's "challenge" have been met), so that the evidence necessarily seems circumstantial. But the vast majority of the evidence is consistent with the view that HIV is a cause (and probably a sufficient cause) of AIDS. How, in these circumstances, can *The Sunday Times* so consistently assert the opposite?

By selective reporting of the evidence, for one thing. Thus it has paid much attention to the handful of cases brought to light in the past year in which there are symptoms characteristic of AIDS, but no HIV infection. But it is no part of the "HIV hypothesis" that HIV is the sole cause of immune suppression. Similarly, it has reported that infected haemophiliacs treated with purified blood-clotting factor VIII have benefited from an improvement of circulating CD4⁺ T-cells (supposedly diagnostic of progression to AIDS) without acknowledging that this may also be consistent with the maligned hypothesis.

More seriously, and much worse, it has reported that deaths in West Africa supposedly due to AIDS are attributable to malaria and tuberculosis instead, neglecting evidence to the contrary previously offered to it, and has asserted that AIDS is not a serious problem in Uganda, in East Africa, on the strength of the opinions of two French health workers without mention of the opinions of public health officials with access to evidence to the contrary. It has repeatedly drawn attention to the "myth of heterosexual AIDS" on the grounds that the spread of AIDS has been less rapid than predicted in the early days — and without allowing for the possibility that public education may have had some effect.

Imbalance

There is more obvious evidence of unbalanced editorial practice. Thus at least one critical letter from a researcher in the field sent to *The Sunday Times*, and which deserved at least partial publication on the grounds of fairness, was not used. Another researcher complains that several hours spent in conversation with Hodgkinson might never have taken place, to judge from the following week's reporting. And there are dangers, of course. Who is making the "terrible

blunder, costly in both money and lives..."? The "scientific establishment", as it is called, or *The Sunday Times*? Impetuously, the newspaper seems not to have considered how much damage it will have done by recruiting young and adult people to the ranks of those who consider the discomforts of "safe sex" to be tiresome encumbrances.

That is where the issues of press freedom enter. The public interest requires that *The Sunday Times* should not follow its perverse line on the causation of AIDS, but that would entail censorship, for which there is no mechanism and which is, in any case, a greater evil. Nor could the Press Complaints Commission help; that is a voluntary association of British periodicals that adjudicates on complaints against editorial decisions, but which is necessarily concerned with individual grievances. What teenager will complain at the encouragement now on offer not to bother with condoms? On the face of things, there is nothing to be done. It is as if the newspaper had taken it into its head that the British prime minister should be replaced (which happens also to be true).

So how, in an open society, is a newspaper to be discouraged from following a line that is seriously mistaken, and probably disastrous as well? Compulsion is out of court. Reason and ridicule in some proportion may help, but there are limits to the quantity of such material, however entertaining, that an international journal such as this could foist on readers outside the United Kingdom. A boycott of sales of the newspaper would be ineffectual; newspaper readers' habits are not easily understood. Picketing the newspaper's offices, in a distant part of London, would be impracticable.

Remedy

Nature therefore offers the following device, which can be only a small part of a remedy. Each week, the coverage of AIDS in *The Sunday Times* will be reported as if it were news, and in enough detail to let readers judge whether the newspaper's line on HIV and AIDS shows signs of being modified. Letters of protest to the newspaper will also be considered for publication in *Nature* provided that they are judged suitable for publication in a newspaper such as *The Sunday Times* and provided that they have first been submitted for publication there and have been refused. (Copies of any correspondence arising would be appreciated.)

On the face of things, giving further publicity to the newspaper's line on HIV and AIDS may seem fated to have an effect opposite to that intended. (In a different context, Lady Thatcher, when she was prime minister, used the phrase "the oxygen of publicity".) But there is no danger that *Nature* readers will be seriously misled by second-hand (and necessarily brief) accounts of reports and articles appearing in a single British Sunday newspaper. Indeed, a service of this kind may even enable readers with a macabre interest in what *The Sunday Times* is saying about AIDS to keep the weekly purchase price (£0.90) in their pockets. Sadly, the overlap between the circulations of the two periodicals is too small for that to give the newspaper pause, but it will be a different matter when more is known of the pathogenesis of AIDS in people infected with HIV. That may not be so far off. What will *The Sunday Times* do then? □

Yeltsin loses scientists' support in swing to opponent Yavlinsky

Moscow. A substantial number of Russian scientists, disenchanted by the failure of President Boris Yeltsin to deliver on his promises of support, have decided to put their weight behind one of his leading challengers, the reformist economist Grigory Yavlinsky, in next Sunday's elections.

Until recently, the scientific community had been among Yeltsin's strongest supporters, providing him with significant backing in his power struggles with the former president Mikhail Gorbachev. Many, for example, were active in setting up the Electors Club of the Academy of Sciences (ECAS), a body originally set up to run the successful campaign of physicist Andrei Sakharov as a people's deputy.

This time round, however, the organization is split down the middle in its allegiance. While half of its members continue to support Russian Choice, the pro-Yeltsin bloc headed by vice-premier Egor Gaidar, an equal number have been campaigning actively on Yavlinsky's behalf.

Those who have deserted the Yeltsin camp claim that their decision is based less on disagreements over the specific policies being offered for science (those promoted by Gaidar and Yavlinsky are remarkably similar) than on their confidence in the individuals involved.

Alexei Zakharov, a candidate for the State Duma (the lower chamber of the future two-chamber parliament) points out that there is a wide range of political views in the scientific community. But he says that active support from members of ECAS played a key role in obtaining the 10,000 signatures needed for Yavlinsky's nomination.

The disillusionment of the scientific community with the Yeltsin administration has been growing rapidly in recent months, explained partly by what is seen as the deterioration of the social standing of scientists. Two events in particular have shaken the scientific community's loyalties.

One was last spring. On the eve of the referendum, Yeltsin solicited the support of the scientific intelligentsia and at the same time issued a directive that would have almost doubled their salaries. But after he won the referendum, Yeltsin issued a decree virtually cancelling the directive.

The second event was the October uprising. Most (but not all) scientists supported Yeltsin's dismissal of the Russian Parlia-

ment. But many believed the shoot-out at the Russian White House was completely unnecessary.

In next Sunday's elections, four separate blocs — including those headed by Gaidar and Yavlinsky — have promised to introduce policies to boost Russian science.

Not all scientists are disenchanted with Yeltsin. Vladimir Kadyshchevsky, chairman of the recently created Union of Russian Scientific Communities (URSC), and director of the Joint Institute of Nuclear Research in Dubna near Moscow, argues that Gaidar's Russia's Choice should receive support from scientists.

Kadyshchevsky says that he reached this conclusion after meeting Gaidar, whom he describes as "a serious politi-

cian who understands the need to save science in Russia, and that neglecting science today means facing severe consequences in the future."

Kadyshchevsky is hoping that his meeting with Gaidar will help ease some of the problems facing the scientific community.

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Grigory Yavlinsky: a growing following.

Alexander Zemlianichenko/AP

For example, many research institutes are facing electricity bills which exceed their total annual budget.

But Yavlinsky's supporters, who admit that their candidate's bloc is unlikely to win but hope that he may become the main opposition voice in the new parliament, claim that he is more committed to introducing the reforms that Russian science needs.

Zakharov, for example, said in a recent radio interview that scientists, now the most badly paid sector of the economy, were suffering directly from the destruction of the Soviet state as the state had been the only serious supporter of their work.

But there was no way back. Zakharov said that the Yavlinsky bloc was proposing that the current government policy of selective support for scientists — which means in practice that the allocation of most research money is decided by government officials — should be replaced by a more competitive distribution of funds.

Feasibility studies should be carried out to look into the increased privatization of scientific activity, said Zakharov. And he also said that research scientists who did not meet international standards should be retrained with government help to equip them for other tasks.

Vladimir Pokrovsky

NIH acts to reduce discrimination

Washington. The US National Institutes of Health (NIH) will shortly fill four senior positions with women or non-whites as part of its drive to counter alleged discrimination in its recruitment practices, according to Ruth Kirschstein, the deputy director.

This move follows recurrent accusations of racial and sexual discrimination against the NIH over employment practices at its campus in Bethesda, Maryland.

Kirschstein revealed the four pending appointments — each at the 'senior executive' level, and as such subject to confirmation by Donna Shalala, the secretary of health — in an interview following a meeting last week at which NIH's senior academic advisers had been told that the biomedical research body must rely on action, not words, to counter the allegations.

"If you made one appointment at a senior level for every task force you've set up, your problem would be solved," Clifford Alexander, a lawyer who has advised NIH on improving its equal opportunities strategy, told the director's advisory council.

Kirschstein acknowledged that discrimination had given the NIH unfavourable publicity, and that much of it was deserved. She

pledged to change the institute's culture. "NIH is here to do research, and some people take the view that they don't want to do anything that will slow down the research," said Kirschstein. "That has to change."

Two facts have been highlighted as evidence of discrimination at the NIH. One is that although two-fifths of biomedical researchers qualifying from US colleges are women, they hold few senior posts at the institutes. The second is that about half the staff who carry out menial jobs at the Bethesda campus are black, but only a small proportion of even junior researchers are.

If any of the predominantly white and male advisory council felt that the issue was being exaggerated, they kept it to themselves during a lengthy discussion which dominated the council's first session under new director, Harold Varmus.

Current plans to reduce discrimination at NIH include expanding its equal employment opportunity office, enabling the office to take monthly progress reports from each of the 26 institutes. Sanctions will in future be imposed on managers found to have victimized those who complain about discrimination.

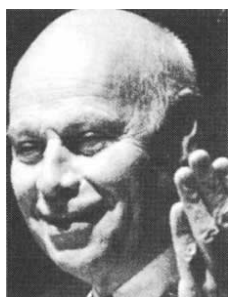
Colin Macilwain

Funding problems loom for new German institutes

Munich. Two more research institutes are to be opened in eastern Germany by the Max Planck Gesellschaft, the body which already runs 65 basic research institutes across the country. This will bring the total number of institutes in the new *Länder* to seven.

But Hans Zacher, president of the MPG, says that future expansion in eastern Germany could face difficulties if the funding mechanism set up to support it, due to expire at the end of 1994, is not extended.

At the beginning of next year, an institute for plant physiology will open in Potsdam, and one for the history of science in east Berlin. A further four — in neuropsychology, theoretical biology, the enzymology of peptide binding and gravitational physics — will be set up over the next two years.



Zacher calls for extension of subsidy.

The big question now is how these new institutes will be funded. In the 11 'old' *Länder*, Max Planck institutes are supported equally by the federal and regional governments. Before reunification, all the money was pooled, so that the MPG was able to divide its money between institutes in different *Länder* free from political pressures.

Since reunification, the MPG's money has been divided into two pools, one for the east and one for the west. This has meant that the new *Länder* have not been required to contribute towards established institutes in the west, and have therefore been able to ensure that their money was being used to

build up a research base in the east.

But if the situation reverts — as planned — at the end of next year, the new *Länder* will have to pay far more. At present, despite new openings, they still have proportionally far fewer institutes than the old *Länder*, and only pay for these. If they were thrown into the general pot, they would have to help pay for the western institutes as well.

Zacher claims that this would be unfair, and has asked the old *Länder* governments to accept the current system until at least 1996, when their expansion programme is planned to be complete. But western *Länder* have proposed a compromise under which the new *Länder* would make up part of the difference between their current contribution and that which will eventually be due.

The MPG has been criticized by other research organizations for its relative slowness in establishing itself in east Germany.

The society denies the charge, pointing out that by the beginning of last year it had established 28 university research groups.

Its goal has been to help reintegrate research into universities of the former East Germany, whose Communist government had wanted to separate research from teaching. But this too is running into problems.

The agreement between the MPG and the universities required the universities to offer staff contracts to group leaders and some other researchers when the five years of MPG support runs out. But the groups have become too large for the universities to absorb all their members.

Zacher has said that support for scientists who have not been taken on to university payrolls will be extended until their projects are completed.

Allison Abbott

Eastern Europe media accused of 'ignoring science'

Geneva. The collapse of communist regimes in eastern Europe has led to a dramatic decline in the serious reporting of scientific topics in print and television media, a meeting of more than 200 science journalists at the European Laboratory for Particle Physics (CERN) was told last week.

To counter this trend, journalists from eastern Europe urged their Western colleagues to give greater publicity to the work of scientists from the former communist countries. Only in this way would scientists receive attention from their own media.

Under the communist regimes, science journalists were strongly supported by the state, partly because political ideology stressed the importance of science education. Extensive science coverage in communist newspapers, radio and television attracted a wide and loyal following; one reason, according to Jiri Grygar, the presenter of a Czech science television show, is that science was seen as the only area of truth in a sea of political propaganda.

Today, however, science journalists find it even harder to convince editors in the new market economies of the value of reporting scientific news — particularly if the science is carried out in their own countries — than their western counterparts.

Viola Egykova, from *Moskovskaya Pravda*, says that 'pseudoscience' has taken the place of real science. Editors find plenty of space for "the speculations of sorcerers, astrologers and phoney prophets", she says. One explanation for this popularity, says István Palugyai from the Budapest-based newspaper *Népszabadság*, is that because the parasciences were forbidden in communist times, people believe they must be true.

Allison Abbott

Survey shows concern about biotechnology continues to grow

Paris. Public support for biotechnology has dropped sharply in member states of the European Union (EU) over the past two years, a European Commission survey has found. But the so-called Eurobarometer survey of 12,800 Europeans also shows that the majority remain optimistic about new technology in general.

More than 80 per cent of those questioned thought that telecommunications and solar energy would make life better over the next 20 years. Almost three-quarters felt similarly about information technology and new materials, but only 42 per cent about space exploration.

The pattern is consistent with the findings of the previous survey, carried out in 1991. Optimism has dropped slightly, per-

haps due to a perception that unemployment in Europe is rising as a direct consequence of new technology's destruction of jobs.

Two years ago, 71 per cent thought that biotechnology and genetic engineering would make life better. Today only 65 per cent do. Moreover, 20 per cent feel these areas will make life worse, compared to only 16 per cent in 1991.

The fall is even more marked in Germany, where only 45 per cent think biotechnology will improve life, compared with 59 per cent in 1991. The proportion of those who think it will make life worse has jumped from 16 to 28 per cent over this period.

The survey reveals that public awareness of the risks of various biotechnological applications has increased in all fields except

animal breeding, where it was already high. Moreover, the increase is linked to reduced willingness to support research, apart from medicine.

Most of those questioned, however, agreed that biotechnology research is "worthwhile and should be encouraged" (except for research on farm animals, and to a lesser extent, on food). More than three-quarters believe that ethical guidelines are needed.

Mark Cantley, head of biotechnology at the Organization for Economic Cooperation and Development (OECD), says that the results from the Netherlands are particularly disturbing. Public acceptance has continued to fall despite a highly organized information campaign on biotechnology.

Declan Butler

Montagnier's AIDS report covers all angles

Paris. Many scientists who had expressed surprise last May when the French government commissioned Luc Montagnier of the Institut Pasteur to assess the French attack on AIDS were comforted last week when the two fat reports that Montagnier presented to prime minister Edouard Balladur emerged as a comprehensive review containing sensible — if unsurprising — recommendations.

While the French media worship Montagnier, and politicians appear to trust him, many scientists remain sceptical of his scientific ideas. Some had feared that Montagnier would use the responsibility given him by the government to promote his own hypotheses, for example that cofactors such as mycoplasmas increase the pathogenicity of AIDS. Others that he would use the opportunity to settle old scores.

In the event, Montagnier did neither. Indeed, only one-tenth of the report deals with research. The rest deals with prevention, health-care, training, and the particular problems of drug addicts, prison inmates and women. To cover these areas, Montagnier consulted widely, giving rise to speculation that, at 61 years of age, he may be angling for a role as national AIDS coordinator.

The report urges the government to create an interministerial committee to coordinate all its efforts against the epidemic, including research. At present, the health ministry hands out disposable syringes to drug addicts at public points, while the ministry of interior's police often handcuff addicts a few minutes later for possession of syringes.

Montagnier criticizes the National AIDS Research Agency (ANRS), set up in 1989, as a "ghetto" with insufficient links to other areas of scientific research, or to practical AIDS programmes. His remedy is to involve the ministry of health directly in ANRS affairs, and to integrate basic and clinical research by creating joint centres.

ANRS's research portfolio is too conservative, says Montagnier. He wants it to support a greater variety of approaches, and to engage in "permanent brainstorming". To this end, he suggests that Jean-Paul Levy, the agency's director, should consult a panel of scientific advisers on all important decisions, and that the agency should create a special fund of FF50 to FF100 million (US\$8.5 to US\$17 million) to support non-mainstream AIDS research.

Montagnier proposes that in the medium term ARNS should be absorbed into the national medical research organization, INSERM, a move that would be possible once INSERM becomes used to administering strategic research. Another recommendation is that all patients should be given access to promising drugs before these are

approved. And he suggests an investigation into illegal clinical trials.

He also suggests setting up an Institute of Complex Infectious Pathologies to combine research on AIDS with that on other diseases involving infection and the immune system, for example ageing and cancer.

Many scientists and physicians have

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Spreading the word: a 'condom' in Place de la Concorde.

welcomed Montagnier's report as a timely

and common-sense review. But some criticize its lack of concrete proposals. Levy, for example, says that his agency already seeks out new avenues of research, and that the proposed advisory structure would simply "formalize" existing committees.

Similarly the patient group, AIDES, criticizes the report for focusing too much on catching up with the current situation, rather than providing the government with proposals for what might happen in the future. The group is also concerned that an interministerial committee might complicate and slow down decision-making.

The government is expected to reply to Montagnier's 49 recommendations in January. But it has already benefited from the report. Although scheduled for release earlier in November, it was held back until 1 December, World AIDS Day. As a result, extensive press coverage of the report coincided with visits by ministers to hospitals and AIDS action groups, providing the government with a unique opportunity to demonstrate its concern for the welfare of patients.

Declan Butler

Czech academics get temporary contracts

Munich. In a move designed to speed up the removal of university faculty members appointed for political rather than intellectual reasons, the Czech government has placed all academics on temporary contracts. They will have to reapply for their posts next year.

The government's move has been supported in principle by the Czech committee of university rectors. But the committee is challenging the way in which the rule is being put into effect, claiming that academic freedom is undermined by the government's role in choosing members of the reselection committees.

The current higher education law, passed in 1990 immediately after the collapse of communism, already allows individual universities to introduce competitive evaluations to check the academic qualifications of their staff.

But according to Josef Splichal, head of the university department in the Ministry of Education, many universities have failed to use this procedure effectively, and there has been little turnover of staff. Splichal also says that many academics chose to accept temporary contracts rather than face selection committees.

Shortly before the summer recess, an amendment to the legislation was hurriedly passed by the Czech Parliament placing all remaining permanent university staff — amounting to two-thirds of the 13,000 teachers and researchers — on temporary contracts. These contracts expire at the end of next September, and before then all those

affected will have to re-apply for their jobs.

A national competition for choosing professors was launched by the ministry last week. Applicants will be judged by committees made up of ministry representatives and academics selected from nominations submitted by the universities. After the appointment of professors, selection committees will be set up for other academic positions.

Martin Cernohorsky, president of the rectors' committee, accepts that the new procedure is needed to accelerate post-communist reforms. But he strongly disagrees with the way it is being implemented and has written to the ministry, suggesting that university autonomy be retained by letting rectors and deans appoint their own selection committees. Each committee could include a representative from the ministry to observe that procedures were being carried out correctly.

But the ministry has so far refused to accept this suggestion. Splichal claims that, as universities are allowed to nominate candidates for the central selection committees, their autonomy has not been unduly threatened in what he describes as a "transient and unstable period".

University staff will be kept on temporary contracts at least until a new law on universities, now under discussion, is passed. But even under the new law, some of them may not be given permanent positions. And in any case the law is unlikely to be ready, as originally hoped, by next summer.

Alison Abbott

US science education reforms 'still lack proper evaluation'

Washington. Ten years ago, a national report found that the United States was in danger of losing its competitive edge over other nations if action was not taken to improve its educational policy. A new report has found that although the government has made some headway in strengthening its role in science, mathematics, engineering and technology (SMET) education, much remains to be done.

The report, published last week under the title *The Federal Investment in Science, Mathematics, Engineering, and Technology Education: Where Now? What Next?* warns that the nearly 300 federally funded core programmes in these fields continue to be burdened by a lack of coordination, evaluation and accountability.

"Unfortunately, much of what was called for in that earlier report remains unfinished", says Karl S. Pister, chancellor of the University of California, Santa Cruz, and co-chair of the new report's 15-member panel.

The panel was convened by the committee on education and human resources (recently renamed the committee on education and training) of the Federal Coordinating Council for Science, Engineering and Technology, or FCCSET.

Federal government spending in the area of evaluation was found to be woefully inadequate: funding for this activity constitutes less than one-half of one per cent (or US\$8 million) of the US\$2.2 billion spent as a whole by the 13 federal agencies that operate core SMET educational programmes.

Only one in five programmes has been fully evaluated; almost half have been neither evaluated nor monitored. Given the present climate of fiscal belt-tightening, the federal government can no longer afford the luxury of "investing in programmes that don't work", says Mary Budd Rowe, a professor of science education at Stanford University, and co-chair of the panel.

Although much of what is contained in the report is not new, one of its main recommendations is that federal agencies with programmes in SMET education should view and manage those programmes much like any "portfolio of investments". As such, the panel says agencies should place greater emphasis on coupling an assessment of educational needs to the unique capabilities of the various agencies.

Furthermore, the panel says that programmes must be evaluated more rigorously and the information "marketed" more aggressively so that teachers, students and researchers are kept abreast of new research findings and are better informed about the availability of new educational materials.

Diane Gershon

Genetics test report urges moratorium on disclosure

London. Individuals should not be expected to divulge information about their genetic history to insurance companies unless it relates to a known family history of genetic disease, or they are applying for an unusually large policy, according to a report published in London this week by the Nuffield Council for Bioethics.

The report is the first to be published by the council, which was formed in 1991 to consider ethical issues presented by advances in biomedical and biological research in the United Kingdom.

A working party set up by the council on genetic screening recommends that the government and the insurance industry should begin discussions soon about the future use of genetic data. In the meantime, it says, there should be a temporary moratorium on requiring disclosure of the results of genetic tests.

Brian Sharp of the Association of British Insurers says that at present no genetic testing is carried out for insurance purposes. "In a sense there already is a moratorium," he says. But even though insurance companies do not ask applicants if they have had a genetic test, they expect to be informed if a test has been carried out.

The report says that individuals should not be required to do so. "In the case of genetic screening, where there is still a lot of anxiety and fear, a genetic test result should not have to be divulged to insurers," says Dame June Lloyd, head of the working

party, who was formerly Nuffield professor of child health in the University of London.

Sharp says that although insurance companies have not debated the issues in depth, they are unlikely to oppose proposals concerning the confidentiality of results from random screening programmes. But insurers are also quick to point out that results of specific genetic tests can be to a potential policy holder's benefit.

Disclosure of test results to family members also raises the question of confidentiality. The difficulty here arises from the fact that unlike normal medical records, the results of genetic tests can have a direct, and possibly detrimental, effect on those related to the individual tested.

In this case the report says that individuals have a responsibility to tell family members of results of genetic screening. Health professionals should receive guidance on how they might be persuaded to do so.

In all other cases including disclosure to employers, however, an individual's rights should be paramount. Appropriate safeguards to maintain this confidentiality should be set up by anyone holding results of genetic screening, and the Department of Health should issue guidelines to this effect, says the report.

"The main message is that we hope that government will set up what we call a coordinating body to ensure that these things happen before screening programmes are set up," said Lloyd.

Other recommendations of the UK report, which is broadly in line with a similar report published recently by the US Institute of Medicine, include the provision of adequate counselling for anyone invited to enter a genetic screening programme before, both during and after testing.

Fiona Gammle



Lloyd: test results should remain secret.

Royal Society calls for electronic archive

London. Scientific research could be damaged unless the UK adapts to the changes in funding of scientific publishing and academic libraries, warns a report published last week. A government-funded national electronic archive is one possible solution to the problem.

A report prepared by The Royal Society in conjunction with the British Library and the Association of Learned and Professional Society Publishers says that funding changes mean scientists must take tighter control of the amount of electronic information they send and receive.

The proposed electronic archive would be in the national interest, it says, because

it would ensure results were accessible to future generations. It could be set up as part of the British Library, and would go some way to preserving all scientific publications as the range of topics become too wide for individual libraries to hold them all.

Other recommendations include routine training and funding provision for academics in the art of retrieving information.

But the report stresses the continued benefits of meeting face to face, rather than relying on electronic communication. And it says that academics should be encouraged to reduce their prose and keep research papers succinct — bringing a whole new meaning to the threat of cut-backs. □

Heads of state to decide on EU research funds

Paris. The next five-year Framework research programme of the European Union (EU), scheduled to start next year, is hanging in the balance this week after the council of research ministers failed to agree at a meeting in Brussels on Monday to its proposed budget of ECU13.1 billion (US\$14.8 billion).

The failure could mean that the long approval process may have to be repeated in 1995. Elections to the European Parliament will be held next June, and the EU therefore only has a short window in which to complete the complex approval procedure if the budget is to receive endorsement from the present Parliament (see *Nature*, 365, 775; 1993).

In a last-minute bid to salvage the programme, the meeting therefore accepted a proposal from Belgium — the current president of the council — that the budget question be resolved by Europe's heads of state, who are meeting in Brussels today (9 December) and tomorrow.

The research ministers did agree that almost 90 per cent of the budget be spent on a research and development programme in seven categories: information and communications technologies (28.2 per cent), industrial technology (16 per cent), environment (9 per cent), life sci-

ences (13.1 per cent), non-nuclear energy (18.15 per cent), transport (2 per cent) and socioeconomics 0.85 per cent).

They also agreed that the remaining money should be divided between international cooperation (4 per cent) and human capital and mobility (6.2 per cent). Despite increasing calls for the EU to spend more on technology transfer, the ministers demanded a cut in the 4.6 per cent of the total which the commission proposed for this area to 2.5 per cent.

Observers are optimistic that Europe's heads of state will agree to a figure close to ECU13.1 billion currently being proposed on Friday. But the move confirms that although the research ministers, such as Paul Kruger of Germany, generally support the commission's proposed budget, their hands appears to be tied by their finance ministers.

Optimism is partly due to the fact that research has moved up the European agenda. In particular, this week's summit is being held to discuss a policy document from Jacques Delors, the president of the commission, on employment, growth and competitiveness, which gives considerable importance to the support of research and development.

But even if the heads of state do agree

on a budget for the Fourth Framework, the programme may nonetheless be delayed, as the research ministers also failed to agree on the proposed ECU1.14 billion funding for the EU's four Joint Research Centres (JRC).

Member states have long criticized the JRCs for their relatively low productivity. Together with the European Parliament, several states have argued that it is "entirely illogical" for the JRCs to be given money before it is decided what work they are going to do with it. They want the JRCs to bid for a slice of each programme like any other research group.

Conflict on this issue was not expected at Monday's meeting of research ministers. The parliament had already accepted a compromise by accepting that the JRCs be funded in the Fourth Framework on condition that they are evaluated before the fifth.

The research ministers, however, said on Monday that they want to tackle the issue now. They are pushing for a different outcome, under which the JRCs would receive some money up front to carry out activities which they are in a unique position to address, but will be required to bid with others for any other research funds.

Declan Butler

China learns cautionary tale from Mongolian 'fossils'

Beijing. Chinese geologists are facing up to the implications of finding out that the discovery three years ago of a huge fossil belt in the far north of the country turns out to have been based on the misinterpretation of geological samples.

The disputed discovery was announced in 1990 by Wang Dong-Fang, Liu Xiao-Liang and other researchers at the Chinese Academy of Geological Sciences' Shenyang Institute of Geology and Mineral Resources. In two published papers, they claimed to have discovered fossilized remains of a new fauna, which they named Qinghezhen, in a 200-km-long belt in a region known as the inner Mongolian land axis.

Calling the fossils "small shelly fossils" or "small shells", the authors claimed that the fauna dated from the Precambrian era. They also concluded that the inner Mongolian land axis had not always existed as a continuous land mass, a conclusion which, if confirmed, would have overturned all previous thinking about the geological evolution of northern China.

Soon after the publication of the two papers, various geologists, including three from the Shenyang Institute, raised doubts about the authenticity of the

reported fossils. But rather than going back to check — or answering questions raised by their critics — the authors of the original papers published two more, drawing further conclusions.

Such actions only increased the determination of critics to find the true nature of the objects in question. Chen Menge, a research professor with the Chinese Academy of Sciences' Institute of Geology in Beijing, decided to look closely at what had happened when geological specimens collected by the scientists were treated in the laboratory.

According to a report of his investigation, published in the journal *Scientia Geologica Sinica*, Chen concluded that

the so-called fossils had been formed during this treatment. He points out that precipitation of minerals in the solution surrounding air bubbles can create fragments that look like fossils.

The final verdict was passed April this year at the annual meeting of the Chinese Palaeontology Speciality Committee. The fossils were certified as false, and the conclusions that had been drawn from them were nullified.

Simon Conway Morris, reader in evolutionary palaeobiology at the Department of Earth Sciences at the University of Cambridge, England, suggests Western scientists should not be too censorious. Chinese palaeontologists, he says, have made good progress with unravelling the story of early skeletal fossils from the Cambrian era, but the inner Mongolian fossils "aren't the first victim of sudden revision."

"In the past supposed Cambrian microfossils from China have been shown to be modern seeds, and the current state of taxonomy is still pretty ramshackle," says Conway Morris. But, he adds, "even if Wang Dong-Fang and his colleagues have made a mistake, it is high time the more complex tectonic belts were searched for early fossils."

You Qin Li



Taiwan to boost biomedical research

Taipei. Taiwan is creating a new medical research organization that will be run along similar lines to Britain's Medical Research Council and the US National Institutes of Health. It will be staffed largely by Chinese medical researchers drawn back from the United States.

Cheng-Wen Wu, director of the Institute of Biomedical Sciences of Academia Sinica in Taipei, is leading the creation of the new organization called the National Health Research Institutes (NHRI). He has already looked at several potential sites for NHRI, which will run an extramural grant programme and carry out intramural research.

Wu says the government is giving full backing to the organization, which will have between 500 and 1,000 researchers in its first five years of operation, because of the success of his own institute. Since its establishment six years ago, this has grown into the largest research institute in Academia Sinica with 400 staff, including more

than 300 scientists.

But some other institutes in the academy are uncomfortable with such rapid growth. Thus although Wu's institute will take a leading role in helping to establish the new organization, NHRI will be independent of Academia Sinica.

It will be an autonomous non-profit organization run by a board of trustees that will include the chairman of the National Science Council (NSC), the president of Academia Sinica, and the ministers of health and education, as well as medical researchers from Taiwan and overseas.

The NHRI plans to have multiple sources of funds, but the health ministry will be the biggest provider. An extramural grant programme started this July with 40 centres of excellence in hospitals and universities each receiving up to NT\$50 million (\$1.8 million) a year, and 20 outstanding investigators up to NT\$5 million each.

Unlike other grant programmes in Tai-

wan, which have an approval rate for applications of about 80 per cent, the NHRI programme makes use of a thorough peer review process, including referees from the United States, and only about 20 per cent of applications are funded. Wu expects the extramural programme to have a budget of about NT\$500 million (US\$18.5 million) in its first few years of operation.

Eventually Wu wants to build several institutes on a site of 50 to 100 acres. But the intramural programme will start next July, before the establishment of any institutes, with funds of about NT\$300 million for six research groups: ageing research; environmental toxicology and occupational disease; health care policy; biotechnology and pharmaceuticals (including clinical trials); medical engineering; and Chinese traditional medicine. Once a sufficient 'critical mass' of researchers is gathered, various institutes will be established.

The new organization will recruit most of its researchers from the United States. Wu, who returned from the United States six years ago, but only recently gave up a post at the State University of New York, Stony Brook, is one of the founding members of the 2,000-strong Society of Chinese Bioscientists in the United States. He reckons there are about 5,000 Chinese bioscientists in the United States and he says people are already lining up at his door.

Some Taiwanese biologists, however, are concerned that the new organization will be a drain on limited resources, and that basic research in biology and medicine will suffer. The government last year started a US\$190-billion six-year national programme for the improvement of infrastructure and roads that has cut into budgets for science.

David Swinbanks

WHO plans guidelines for genetics research

Paris. Hiroshi Nakajima, director general of the World Health Organization (WHO), last week proposed that the organization establish a set of international guidelines for research in genetics and biotechnology, as well as for its social applications.

Giuseppe Benagiano, director of the WHO's Special Programme of Research, Development and Research Training in Human Reproduction, says all laboratories funded by the WHO are likely to be required to observe the guidelines. He adds that the WHO's reputation should ensure that most laboratories outside its jurisdiction also observe them.

The WHO's executive board will decide in January whether to go ahead with the plan, which would start with a broad consultation with the scientific community. **D. B.**

Chemist tipped for top academy post

Taipei. A group of 50 leading Taiwanese academics will meet in Taipei on Saturday (11 December) to select three candidates for the presidency of Academia Sinica, Taiwan's leading organization for government research.

This is a rare event, because presidents are appointed for life. The most likely candidate for the post is Nobel prize-winning chemist Yuan Lee of the University of California at Berkeley. Another possible candidate is Ying-shih Yu, a professor of Chinese history at Princeton University.

The final choice will be made from three individuals nominated by the academy to the president of Taiwan, T. H. Lee. The academy president will have to oversee 21 institutes in science and the humanities, with an annual combined budget that has increased ten-fold over the past decade to US\$110 million, but which is now being squeezed by other government priorities.

Wu Tayu, an 87-year-old physicist who has been president for the past ten years, decided to retire after harsh questioning over the budget for the academy by opposition members in the legislature earlier this year. Academy officials deny reports that Wu's retirement is directly related to his failure to persuade Taiwan to contrib-

ute to the US Superconducting Super Collider (SSC) project. But others feel it may have been a factor.

The academy has experienced rapid growth over the past decade. Seven new institutes have been founded — for Earth science, information science, statistical science, atomic and molecular science, molecular biology, biomedical science and computing — and the number of researchers has doubled, to about 900.

But the academy's budget has been squeezed in the past year as the government embarks on an ambitious six-year plan to improve roads and other infrastructure. The new president will have a difficult task on his hands because there is a perception in government circles that too much effort is put into basic research and producing publications and not enough on practical research of immediate use to society.

The 50 members of the academy's council, which includes approximately equal numbers from the three areas of mathematics and physical sciences, life science, and humanities and social science made up of the heads of the academy institutes and members from overseas, will nominate three candidates, usually one from each of the three main fields.

Yuan Lee is the most frequently mentioned potential candidate. He is one of the members of the council, and has already decided to return to Taiwan in January, whether or not he is elected president, to work as a 'distinguished fellow' in the new Institute of Atomic and Molecular Sciences.

David Swinbanks

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Lawrence Berkeley Laboratory

Lee: likely candidate.

UK protects science base, but cuts overall spending

London. Britain plans to cut public spending on research and development (R&D) even further next year, especially at the Department of Trade and Industry and the Department of Defence, according to Whitehall officials speaking after the government announced the annual budget last week.

But Kenneth Clarke, the Chancellor of the Exchequer, said that the government would protect spending on the "science base" — which includes programmes funded through the research councils and universities — from the widespread cuts in public spending, in line with the Conservative party's election promise last year to make science a top priority.

The United Kingdom will increase spending on the research councils and other related institutions in the financial year beginning next April by 4.2 per cent to £1.08 billion (US\$1.59 billion), just above the anticipated rate of inflation. It also plans to increase spending the following year by a further 3.5 per cent.

The government will also increase the budget for universities by 6.7 per cent. But it has earmarked most of this money to pay for extra students, and it is unclear how much will go to research.

Nevertheless, officials at the Office of Science and Technology (OST) say that total government spending on R&D will probably continue to fall — it has dropped two per cent in real terms since last year. Official figures are not yet available, because individual departments are still deciding how to divide the lump sums they have been allocated.

William Waldegrave, the science minister will decide later this month how much individual research councils get from the science budget, on advice from the Advisory Board for the Research Councils (ABRC), which meets for the last time on 15 December.

Sir David Phillips, the chairman of the ABRC, describes the outcome of this year's budget negotiations — which were particularly difficult because of the current budget deficit — as "a very good settlement compared to what it could have been".

OST officials say that the coherence of the government's strategy to link science to wealth creation, outlined in the May white paper (policy document), helped them to fend off spending cuts. They had argued that such cuts would sacrifice the initiatives this strategy had generated, and thus threaten Britain's long-term economic prospects.

The pressure group Save British Science said it welcomed the chancellor's decision to protect spending on the science base, but was nonetheless concerned about the drop in total R&D spending. □

Fermat's theorem proves elusive to the last

London. The proof of Fermat's last theorem, having escaped mathematicians for over three centuries, is still proving more elusive than it appeared six months ago, when Andrew Wiles of Princeton University outlined his solution to a packed meeting at the Newton Institute at Cambridge University in England.

At the time, mathematicians hailed the presentation as one of the most significant achievements of the century, although they acknowledged that Wiles still needed to polish some details. But they have since become somewhat frustrated by Wiles' apparent reluctance to release details of his proof, giving rise to speculation that he had encountered an unanticipated problem.

Last Friday, after several months of growing pressure, Wiles issued a statement confirming their suspicions. The statement said that he has not yet been able to complete a key step in the proof, and that the manuscript remains unsuitable for release, since "a lot of work remains to be done".

Wiles also promised to give a full account of the work that he has carried out on the proof when he presents a scheduled lecture course at Princeton in February.

Few mathematicians who heard Wiles' presentation at Cambridge doubt that he has found the right path to a solution. John Coates, for example, professor of mathematics at Cambridge, who helped set up the meeting, says he is confident that it will still work. But he admits that, as things now stand, the proof remains incomplete.

Fermat's last theorem states that there are no values of n higher than two for which the formula $a^n + b^n = c^n$ can be solved in non-zero integers. The French mathematician claimed he had a proof, but that he could not write it down because the margin of the book he was reading was too narrow; modern mathematicians doubt that his proof was valid, and acknowledge a watertight one will be extremely complex.

Wiles has spent seven years developing an approach that uses a range of recent mathematical advances. His presentation at Cambridge needed three seminars, and its written version is said to run to one-thousand pages in draft form.

But Wiles has only shown his preliminary manuscript to a small circle of mathematicians, who are helping him iron out the

final details. This itself has raised eyebrows among some who felt that any problems might be worked out faster if Wiles circulated his manuscript more widely.

Until last Friday, Wiles and his Princeton colleagues had been staying silent about

the cause of the delay, and had ignored letters and faxes from other mathematicians who have offered to help.

His statement now confirms widespread speculation that he has encountered a problem. Much of his proof consists of extending the work of Mateus Flach, like Wiles a doctoral student

at Cambridge who now teaches at the University of Heidelberg in Germany, through a number of complex steps. It now appears that his original arguments justifying one of these steps are insufficient.

"In his Cambridge presentation, Wiles seemed to have found a short-cut, but it appears that this has not worked out," says one mathematician. "As a result, the proof of this particular link may have to be established by more conventional means."

Mathematicians are confident that such efforts will eventually succeed. "No-one has faulted his line of reasoning, or really believes that the link he makes is not valid," says Ken Ribet, professor of mathematics at the University of California at Berkeley who in the 1980s developed some of the techniques that Wiles uses in his proof.

In his statement, Wiles says that a number of problems which emerged during the review process have been resolved. And a key step in the proof, namely that if the so-called Selmer group is small, then every semi-stable elliptic curve over the rational numbers is modular, has been confirmed.

But the complete proof requires the demonstration of a precise upper bound to the number of elements of the particular Selmer group. This Wiles has not yet achieved, although he remains optimistic of doing so. "I believe that I will be able to finish this in the near future, using the ideas explained in my Cambridge lecture," he says.

Nevertheless, mathematicians are remaining cautious about claiming that the theorem has been proved until this work is completed and Wiles publishes his final manuscript. And many are now quoting the words of the Dutch poet Piet Heim: "Problems worthy of attack, prove their worth by hitting back."

David Dickson

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Andrew Wiles outlines his proof in Cambridge; "a lot remains to be done".

Peter Goodard/Science Photo Library

Polish Academy defends itself Canopy network

SIR — I am glad that you take an interest in science in Poland, but must ask you correct a number of misstatements in a recent article (*Nature* 364, 748; 1993).

The State Committee for Science Research (KBN) was established not to "take over from the communist-controlled" Polish Academy of Sciences (PAN), but, in the interests of good organization and the more systematic financing of research, to replace both the State Committee for Science and Technology and the State Office for the Implementation of Science and Technology. There is no question of a transfer of power from PAN, which never enjoyed it.

In my opinion, it makes no sense at this stage to ask which of PAN, the universities and the state-controlled institutes used to be the most tightly controlled, but your article conflicts with the recently published report of the secret services that PAN used to be a haven for scholars expelled from the universities on political grounds.

Indeed, the secret report published in 1984 noted that PAN supported individuals who expressed both theoretical and practical opposition to the regime, "dominated" as the academy was by "hostile elements". (That is the opinion of Jan Krzysztof Frąckowiak, which you do quote.)

Perhaps the best proof of that is the composition of the Polish government before the elections in September. No fewer than four members of the government, including the prime minister, Hanna Suchocka, were members of PAN institutes.

We are proud that during the 40 years of PAN's existence, it held to the principle of conferring membership only on creative scholars, most of them not party members. Many of PAN's delicately expressed opinions were very different from party ideology. It is also relevant that PAN's 80 research institutes, employing only 8 per cent of Poland's scientists, have always enjoyed the highest ratings.

Many of its members have enjoyed cooperation with leading national academies and other scientific societies in 40 countries, enabling them to work in laboratories elsewhere and to carry out joint projects. During the past 15 years, of 4,100 researchers employed in PAN institutes, no fewer than 3,000 were awarded fellowships to attend British research institutes, thanks to agreements with the Royal Society and the British Academy. At the same time, several British researchers are foreign members of PAN.

The future of science in Poland depends on the creative activity of researchers supported by a rational science policy and not, as your article claims, on whether the

State Committee for Science Research will survive another change of government.

Leszek Kuźnicki
(President)

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Testing for Alzheimer's

SIR — Beneath the title of an advertisement in *Nature* (11 November 1993, facing page 129) headed "ApoE predicts Alzheimer" was the quotation "... a common marker for a devastating and frightening disease must provide the impetus for social and legislative change. Our thinking of Alzheimer's disease cannot be the same again" followed by the reference "J. Scott Lancet 342, 1993".

Let me expand on this statement. I also said: "It is not often that a scientific discovery points the need for social change. The finding that a common genetic variant of apolipoprotein E (apoE4) confers a high probability that an individual will develop late-onset Alzheimer's disease is such a discovery ... We have to consider urgently what we should do about such discoveries. The ethical, legal, psychological and social implications of this discovery must force us towards a new responsibility."

I am on the public record (*The Guardian* and the BBC Radio 4 Tonight programme) in stating that I do not believe that this test should be generally used. My reasons are in line with similar recommendations for Huntington's chorea. There is no treatment at present for either of these conditions and guidelines to protect the individual from injudicious genetic testing are not clearly established. Therefore there can be very few indications for performing such a test. Exceptions may be that the test could be valuable in the diagnosis of Alzheimer's, but the test should be performed only with informed consent and counselling of the relatives of a demented individual, because a positive result would have serious long-term implications for at-risk relatives. Also if an individual were to insist on testing because of familial risk, then with counselling, this might be appropriate. With these caveats, I do not believe that this test should be made generally available. Therefore my name should not be used to advertise this test.

James Scott

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SIR — We established the Canopy Research Network (CRN) in July with a two-year planning grant from the Database Activities Program and the Ecosystems Program of the National Science Foundation to establish methods to collect, store, analyse, interpret and display three-dimensional data relating to tree crowns and forest canopies. We are now soliciting input from researchers in other fields who have developed techniques relevant to the complex three-dimensional structure of tree crowns and forest canopies.

Over the next years, we plan: (1) to compile an array of research questions requiring information on canopy structure; (2) to examine useful information models and software tools in use in allied fields; and (3) to develop conceptual models and recommendations for the types and format of information and analyses necessary to answer research questions posed by canopy researchers.

Readers with information about analytical tools that could be applied to this area of research are asked to contact us, either at the CRN e-mail bulletin board at canopy@elwha.evergreen.edu, or at either of the addresses below.

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Modest proposal

SIR — Your leading article "Crime and punishment" (*Nature* 365, 591; 1993) makes the convicting of innocent people for crimes of terrorism seem irrational as well as illegal and immoral. It is not.

If only the guilty are punished, an Irishman in England will be safe if he does not plant bombs. If innocent Irishmen are punished after a bombing, the only way an Irishman can be safe is to get bombers arrested before their bombs go off. This puts pressure on the entire Irish community to be informers.

Random punishment generates the same compulsion to inform as does collective punishment. Such methods appeal to counter-terrorist forces, and maybe they sometimes work, but prominent devotees have come to bad ends.

Charles W. McCutchen

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Science and religion (contd)

SIR — Hermann Bondi is right when he says that “the human mind is singularly liable to be mistaken on religious issues” (*Nature* 365, 484; 1993), but to conclude that the less attention given to religion the better is, I believe, to bury one’s head in the sand. It is true that differences in religious belief have led to severe conflict throughout history, but might this not indicate the importance of the question and the deep desire of the human heart to know God?

Specific criticisms against Christianity often centre on the Crusades or the Inquisition. This argument, that a discipline which can be twisted and misused towards destructive ends should be avoided, is a familiar one. However, if we scientists feel a sense of *déjà vu*, perhaps it is because this is a criticism more often levelled at science itself — the destructive power of gunpowder, nuclear weapons, or fears of genetic manipulation leading to eugenics for example. But just as the bombing of the World Trade Center in New York was a misapplication of the science of explosives, disasters such as that at Waco are due to the gross distortion of selected ideas from religions. That such disasters occur is an indication of our poor spiritual condition and the inability of many to separate what is true from what is not true, being led by the blind because there is no one else to satisfy their spiritual hunger.

Ian Goldby

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SIR — In asking why science was for so long “confined” to non-Christian parts of the world, Bondi seems to suppose that Christianity has changed little over the past two millennia and that it is reasonable to homogenize it into one undifferentiated whole. Yet this is manifestly not the case, as may be seen in the very phenomenon he finds so puzzling, the rise of modern science. The (relative) absence of science in ‘Christian’ Europe before about 1600 may be connected with a general failure of mediaeval Christianity to liberate itself from a-christian categories of thought. When the ‘scientific revolution’ did take place it was a result of a peculiar combination of social, economic and religious changes in the sixteenth and seventeenth centuries. Above all it was the new availability of the biblical text to ordinary people that provided a fresh vision of a wholly demythologized Universe and of a divine mandate to study it. Where that insight prevailed science flourished; elsewhere it generally did not. The Chinese science that developed no deductive geometry or

mathematical explanation of planetary motion lacked a belief in a divine creator constructing the Universe on a rational plan; on the other hand the path-breaking work of Kepler and Newton was informed and inspired by a theism that came straight from the pages of the Bible.

Bondi may not like any form of religion; but he can hardly deny a crucial role to that form of Christianity from which sprang the science that unites us all.

Colin A. Russell

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SIR — Bondi’s arguments are flawed for two different reasons. First, the claim that religious beliefs have long been used as justification for human conflict is undoubtedly true, but a very similar criticism could be made of science itself. For example, in the nineteenth century, science buttressed beliefs on racial inequality with a wealth of ‘data’ based largely on anatomical observations. In the twentieth century scientific discoveries have been crucial in facilitating warfare on a scale and with a ferocity never previously witnessed in human history. Large slices of the national income of technically advanced countries are still consumed in using scientific methods for developing ways of killing people more efficiently. Yet, despite these unpleasant facts, we continue to pursue the scientific enterprise because we also recognize its enormous potential for good. Scientists do not give up science because it is widely misused any more than people give up sex because of the existence of rape. Similarly, given the immense hold that religious belief has on a very large segment of the world’s population, the appropriate response to misuse of religious belief is not to confront religion *per se* but to oppose its misuse.

Bondi also questions the claim that the Christian belief in an ordered Universe provided the necessary background to science, since the ‘home of science’ was confined to the non-Christian parts of the world for so long. But the further question then arises as to why science failed to develop any further in these other parts of the world. It is a fact that the enterprise that we now recognize as modern science — complete with journals, scientific societies and investigative ways of thinking — emerged in Europe in the seventeenth century. And anyone who reads the works of the ‘early modern scientists’ will be struck by their frequent reference to their belief in God as providing a basis and motivation for their investigations. Without doubt religious belief was only

one factor among many that led to the emergence of modern science. Nevertheless, the active involvement of so many Christian theists at this critical period of its development is certainly consistent with the claim that their beliefs provided an impetus that was lacking in other belief-systems and allied social structures.

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SIR — Bondi is correct in saying that theologians differ, but disagreements are common in all fields of human enquiry. For example, not every scientist agreed with steady-state theory.

Bondi and Arno Arrak (in the same issue) are also correct in stating that religion has been misused and perverted, but science has been similarly abused. The Nazis carried out hideous experiments on people in the name of science. Physicists of the highest calibre gathered at Los Alamos to develop weapons of mass carnage. Yes, religion has been perverted at times, but so has science, and that abuse says more about fallen human nature than it does about either religion or science.

Arrak and Michaelene P. Llewellyn (also in the same issue) respectively say that religion is “a product of social evolution” and that “[r]eligious beliefs are rationalizations for various behaviours”. Both statements presuppose that there is no God and that religion is an invention of man. These are hugely biased presuppositions on which to base any study of religion.

They might do well to consider 1 Corinthians 1:19. The scientific claim that matter and life have natural origins is in direct contradiction to Genesis and these are areas where we might look for science to be frustrated.

Thomas P. Scott

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SIR — Josephson (*Nature* 362, 583; 1993) believes that religion can only gain from being investigated scientifically. But do we want it to gain? Would it not be better for mankind if it were to disappear quietly, forever?

The prime function of organized religions is to retain power and influence, in order to continue their suppression of ideas of which the world is in sore need. Science can function only in a climate of scepticism and open-mindedness, a climate in which religion can only compromise itself and, eventually, be explained away. Truly, science is incompatible with religion.

John Noble

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Electronic publishing

SIR — Your editorial comment (*Nature* **365**, 689; 1993) on electronic journals at the Frankfurt Book Fair is timely. As it happens, the general assembly of the International Council of Scientific Unions (ICSU) was taking place at the same time in Santiago de Chile. Progress was reported there on a preliminary study of electronic publishing by members of the ICSU Press (of whom I am one) during 1993.

The ICSU initiative on electronic publishing is particularly concerned with the intellectual property rights in digitally stored scientific texts. A meeting of experts held under the joint auspices of ICSU and UNESCO in Paris last June agreed that there are two major needs: (1) a universally recognized system of bibliographic control of digitally stored scientific texts and (2) a simple method to protect these texts from unauthorized copying or amendment. It was concluded that the time was ripe for scientists and publishers acting together to seek solutions to these.

ICSU Press, at the request of ICSU, has accepted responsibility for monitoring developments in electronic publishing and will endeavour to identify solutions to these problems. The task requires the collaboration of authors, readers and publishers. Although each group has different needs, these are not distinct; indeed, in electronic publishing, the author is often also the publisher as well as the user. However, our meeting of experts, including scientists, lawyers and publishers, as well as executives from WIPO, ICSU and UNESCO, agreed that a common solution must be found if the system of quality control of primary scientific publication is to be protected as electronic journals grow in importance.

We realize that our study is by no means unique, but we may be the first to recognize — and to say publicly — that the matters of concern are common to all interested parties. For this reason, if no other, scientists and publishers should be talking together.

For the present, anybody with a contribution towards solving either of these problems should write to me.

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Military students

SIR — Until last year, unmarried Spanish males under the age of 30 working overseas were exempted from their nine months' military service if they were working abroad for at least three years,

but this exemption no longer applies.

As a result, a 25-year-old Spanish graduate student who has been working with me for the past two years and who still has two years' employment ahead of him has been recalled to do his military service immediately. His work for a doctorate will be in jeopardy and my small group working on anoxia in heart cells will be seriously disrupted. I know among my immediate contacts of two other Spanish biologists, in the United States and in Scotland, who have likewise been summarily recalled by the army.

The change in policy probably results from Spain becoming a full member of the European Communities (EC) and the consequent waiving of work permits for Spaniards by other EC countries.

The international scientific community should be aware that employing single, Spanish males under the age of 30 who do not yet know how to fire a rifle or salute a flag is likely to disrupt their research.

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Mathematics yes, physics no

SIR — The exciting diversity of life, together with the conflicting requirements for both precision and generality, give biologists especial difficulties with scientific terms. Biology, mathematics and physics all have the need for terminology to be both general and precise. Some recent correspondents¹ have advocated a physics-like model for biology. Physics deals with a few concepts of wide applicability, so it can afford the luxury of a limited number of terms, each with restricted meaning — the 'one word, one meaning' convention.

Mathematicians have the same need for both precision and generality but they provide a better model for biologists. A general term can be made more precise in a number of ways for a specific action. A general statement may be about all entries in a vector or matrix; a specific statement may use subscripts to refer to either a specific or a general entry. A proof may relate to, say, any monotonic function and a specific function can be substituted for a particular application. A complex equation with many variables can be differentiated 'with respect to' a specific variable. Each of these three ways allows strong general statements to be given a specific meaning at their time of use.

The desire for a very detailed terminology that covers every eventuality has long been a problem in biology. For

example^{2,3}, any respectable botanical text of the nineteenth century will describe the ten forms of vernation that date back at least to Linnaeus. The texts would include obvolute, equitant, circinal, imbricate, and so on. Most plant scientists would now have to ask what vernation was, let alone know all the modifiers. The terminology is now mercifully forgotten for everyday use.

The philosopher of science, Karl Popper, in his essay 'Against big words'⁴ says: "Every intellectual has a very special responsibility . . . he owes it to his fellow men (or to 'society') to represent the results of his study as simply, clearly, and modestly as he can. The worst thing he can do — the cardinal sin — is to try to set themselves up as great prophets vis-a-vis their fellow men and to impress them with puzzling philosophies. Anyone who cannot speak clearly should say nothing and continue to work until he can do so".

When it comes to terminology, biology does not need physics envy. DNA preserve us from potonouns, naponouns, xaponouns, exaptations, and half-equant induplicate vernation.

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Negative impact

SIR — The News story about a survey of 250 Indian companies by the Centre for Technology Studies (CTS) to assess the impact of economic liberalization on industrial research and development and innovation¹ merely reiterates an acknowledged fact documented in the eighth Five-Year Plan².

Moreover, CTS's support for liberalization is negated by its own findings of a fall in research and development expenditure (from 1.1 per cent to 0.8 per cent), and the number of patents (from 22 to 13) following liberalization. Thus, calling for more governmental support is not only devoid of scientific legitimacy but also at variance with a policy intended to attract such support from outside.

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A dose of molecular medicine

Participants at *Nature's* conference "From DNA to Drugs" in Amsterdam (2–3 December) found that applying molecular biology to medical problems combines daunting challenges with revolutionary possibilities.

Amsterdam. The explanatory power of molecular biology has unexpected ramifications. Introducing his talk on "Protein structure and drug design" last week, Tom Blundell (Birkbeck College, London, and director-general of the Agricultural and Food Research Council) pointed to a residue in the structure of the enzyme porphobilinogen deaminase and remarked that a mutation at this spot was responsible for the acute intermittent porphyria that affected King George III of England. That condition led to fits of madness after overeating, the imposition of savage taxes on the American colonies during a royal fit, the American War of Independence (as it is called in Britain) and thus the formation of the United States itself. As an example of molecular pathogenesis, it could hardly be bettered; but as the rest of the meeting showed, the challenge of turning structural insight into effective treatments remains daunting.

Indeed, before it can even begin, it is generally necessary to find the responsible gene. That task will surely become easier as the vast job of charting the human genome draws to a close, and more than 90 per cent of it will soon be physically mapped (Daniel Cohen, G  n  thon). Even now, though, it is clear that a single clinical entity (such as Friedrich's ataxia) may have more than one cause (Jean-Louis Mandel, Institut de Chimie Biologique, Strasbourg), while different clinical entities (such as multiple endocrine neoplasia type IIa, type IIb and Hirschsprung's disease) can stem from mutations in a single gene, in this case the oncogene *ret* (Bruce Ponder, Cambridge).

Many commoner conditions, including most of the major killers of Western populations, are multifactorial, and pose even more intractable problems. In Europe, a polymorphism in or near the gene for angiotensin converting enzyme appears to be responsible for as many cases of coronary artery disease as smoking (Fran  ois Cambien, INSERM, Paris). The 4.4 form of apolipoprotein E also seems to be a strong predisposing factor, and has an even more drastic effect on susceptibility to Alzheimer's disease. While these findings are highly significant in themselves, they also surely herald major advances in the understanding of the epidemiology of such conditions.

Elsewhere, there are even fewer clues to follow. A decade after HIV-1 was first isolated, it is still not clear exactly what features of an immune response confer protection against AIDS, so that designing an effective vaccine has proved extremely difficult

(Warner Greene, Gladstone Institute, University of California, San Francisco). But a protective response is possible; rhesus monkeys infected with SIV bearing a deletion in the *nef* gene resist subsequent challenge with the native virus, and a small group of San Francisco men have been HIV-positive for more than 12 years without showing any evidence of disease. The hope must be that further study of these cases will eventually allow the design of a vaccine that induces such a response in everybody.

Once the pathogenesis of a condition is clear, the systematic study of therapeutic intervention can begin. But here too, pitfalls abound. The structure of a protein complexed with a potential drug may have to be determined several times before rational design has honed the drug to perfection (Tom Blundell). Similarly, the regions of a mouse monoclonal antibody that determine its complementarity with an antigen are not the only parts that must be retained when it is "humanized", at least if high affinity is to be preserved (Cary Queen, Protein Design Labs, Inc., San Francisco). And the features responsible for efficient targeting of ribozymes, also potential drugs, are only beginning to be understood (John Rossi, Beckman Research Institute, City of Hope, California). Even the seemingly simple oligonucleotide requires multiple chemical modifications before it has the stability and affinity to make a satisfactory reagent (Richard Wagner, Gilead Sciences, Foster City, California).

Nor is it any easier to use reagents expressed by the patient's own cells. Tumour-infiltrating lymphocytes from patients with end-stage cancer can be grown *in vitro* and returned to them, inducing remission in some (Steven Rosenberg, US National Cancer Institute). But success is not common, even when the lymphocytes are made to produce tumour necrosis factor and interleukin 2 to enhance their effectiveness. In other cases, painstaking work with several viral vectors in various tissues has been necessary to obtain satisfactory expression of proteins such as factor IX in mice; even then, success has been difficult to replicate in dogs (Inder Verma, Salk Institute, San Diego). Moreover, even now that mice, at least, can be made to express stable and therapeutically useful levels of a mini-dystrophin gene, questions remain about the safety of the vector and the immunogenicity of the protein (Axel Kahn, Institut Cochin de G  n  tique Mol  culaire, Paris).

On other occasions, of course, immune responses may be turned to good account.

Indeed, vaccine design may be transformed by molecular biology before any other branch of medicine. But even here, there are puzzles. Why must recombinant influenza and vaccinia viruses, each containing a different epitope from the major surface protein of the malarial sporozoite, be given to mice in a particular order to elicit an effective immune response (Fidel Zavala, New York University Medical Center)? Why does naked DNA injected into the skin (preferably with a DNA gun) induce an excellent immune response, when the same DNA administered intravenously does very little, even when complexed with liposomes (Harriet Robinson, University of Massachusetts Medical Center)? And why is the canary poxvirus, which cannot even replicate its DNA in mammals, nevertheless highly effective at presenting proteins to the immune system (James Tartaglia, Virogenetics Corporation, Troy, New York).

Clearly, the gulf between the laboratory and the patient remains deep. But already, the first stirrings of a revolution can be seen. Take, for example, the ability to select from a pool of more than 10^7 random peptides the one that can block the growth of *Staphylococcus aureus*, antagonize only one class of enkephalin receptor or lower blood pressure in mice (Richard Houghton, Torrey Pines Institute for Molecular Studies, California). Will not the search for new drugs shortly be changed out of all recognition?

The power of implanted fibroblasts secreting nerve growth factor to reverse cognitive deterioration in aged mice (Fred Gage, University of California, San Diego) also points to an imminent extension of therapeutic competence. Even the hitherto intractable health problems of the developing world are coming under attack, and the possibility of transforming the BCG bacillus into a cheap, safe and effective vaccine against conditions such as leishmaniasis (Barry Bloom, Albert Einstein College of Medicine, New York) raises hopes that they will benefit as much or more than the wealthy West (or North).

None of these developments is yet in clinical use, and none is free from problems. Indeed, it is now clear that many of the hopes and expectations with which the search for 'gene therapy' began were unrealistic. But that in no way invalidates the medical potential of molecular biology. Rather, it is now plain that the dedication of the pioneers in the field has started an advance that will one day be able to improve the existence of everyone on the planet. **Nicholas Short**

Replicator renaissance

Bruce Stillman

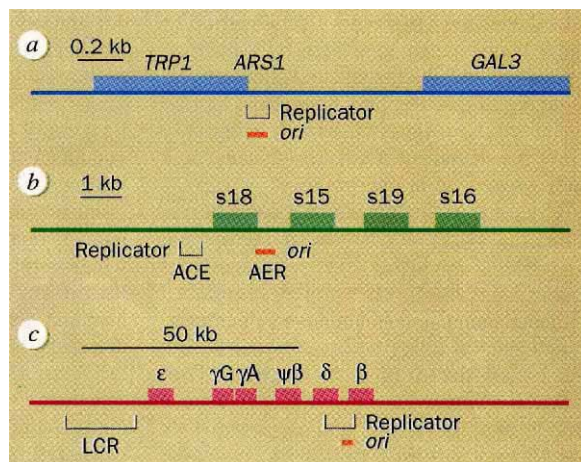
CERTAIN DNA sequences, responsible for initiating a new round of DNA replication, have been well characterized in bacterial chromosomes, plasmids, bacteriophage genomes, in the genomes of many viruses that infect eukaryotic cells and in the chromosomes of some single-cell eukaryotes such as *Saccharomyces cerevisiae*. Not so in vertebrates, where the nature of such sequences has remained controversial¹. An advance that should go some way to settling matters is described on page 588 of this issue² by Kitsberg *et al.* — these researchers have combined genetic and physical mapping methods to locate an origin of DNA replication at a specific location in a human chromosome.

Thirty years ago, François Jacob and Sydney Brenner proposed the replicon model to describe how replication of the bacterial chromosome was controlled³. They envisaged that a genetic element in the DNA, the so-called replicator, would function as a target for a positive-acting initiator protein and that this combination would control duplication of the chromosome. The replicator was proposed to be synonymous with the origin of DNA replication, and hence the term replicator has been lost from the current literature. Many genetically defined replicators and initiators have been characterized in great

detail, including the *oriC* replicator and *dnaA* initiator protein from *Escherichia coli*. Although the details of the replication of the bacterial chromosome are much more complicated than the model supposes, the essence of the replicon model has endured⁴.

Unlike bacterial chromosomes, the chromosomes of eukaryotes cannot replicate from a single origin of DNA replication and so have multiple origins. In cells of the yeast *S. cerevisiae*, replicator sequences were first found by the identification of autonomously replicating sequences (ARS) which enable small plasmids to replicate in the nucleus in synchrony with the cells' chromosomes⁵. Physical mapping⁶ subsequently demonstrated that the ARS sequences are the

origins of DNA replication in the plasmid. Most interestingly, however, only a subset of the ARS sequences function as a replicator in their natural location in the chromosome. This established the principle that there are many more potential origins of DNA replication in chromosomes than are actually used. Moreover, even the active origins are in excess in the



Replicators and *oris* in eukaryotic chromosomes. *a*, Near the *TRP1* gene on chromosome IV of *S. cerevisiae*, *ARS1* is necessary and sufficient for replicator function. The *ori* also maps to the same region. *b*, The *Drosophila* third chromosome chorion gene locus contains an amplification control element (ACE) that is necessary and sufficient for replicator function. In this case, however, the major *ori* does not overlap with the replicator, but is located downstream of the *S18* gene, overlapping a regulatory element that functions as an amplification-enhancing region (AER). *c*, The human β -globin locus contains an *ori* that overlaps with a sequence necessary for replicator function. It is not known, however, if this sequence is sufficient for replicator function. The locus controlling region (LCR) affects replication timing in S phase, but it is not part of the replicator because, in cells where the LCR is not active, the replicator still functions. In each case, the genes surrounding the replicator and *ori* are indicated by the boxes and transcription is from left to right. Note the different scales.

chromosome because many of them can be deleted without a dramatic effect on chromosome stability⁷.

Genetic studies of replicators in *S. cerevisiae* have revealed that they have a complicated, modular structure^{8,9}. The best characterized of them, *ARS1* from chromosome IV (see figure), contains two essential determinants A and B, the B element being further subdivided into three redundant elements⁸. This structure is somewhat reminiscent of the modular structure of eukaryotic promoters and, indeed, one of the B elements in *ARS1* binds a transcription factor. The essential A element binds a multiprotein complex called the origin recognition complex, a strong candidate for the eukaryotic cell initiator protein¹⁰.

To date, the most extensive genetic analysis of a chromosomal replicator in a cell of metazoan origin has been the amplification controlling element (ACE) present in a chorion gene cluster in *Drosophila melanogaster*¹¹. This small DNA element signals the developmentally regulated amplification of the chorion genes in terminally differentiated ovarian follicle cells in the female. The amplified chorion genes make copious amounts of egg-shell proteins. The ACE, when placed at other locations in the *Drosophila* genome, will cause developmentally regulated amplification of the adjacent DNA.

The *Drosophila* ACE is of particular interest because, although it functions genetically as a replicator element, the actual origin of DNA replication is located in a broad, adjacent region. This suggests that the replicator need not correspond to the physical location of the origin of DNA replication, and it may therefore be prudent to revive the term 'replicator' for the genetically defined *cis*-acting determinant that activates initiation of DNA replication. When, on the other hand, physical mapping techniques locate the actual initiation point of DNA replication, and not just the regulatory sequences, the term *ori* (for origin of DNA replication) would be most appropriate. In many cases, the replicator and the *ori* overlap.

The report by Kitsberg *et al.*² may signal the beginning of a renaissance of the replicator principle in mammalian cell chromosomes. These authors studied the replication of the well-characterized β -globin gene cluster in human cell lines. This locus has been extensively characterized in humans because it contains a large number of genetic defects that cause β -thalassaemias. The locus replicates early in the S phase of cells expressing the β -globin gene, and late in the S phase of cells not expressing the gene. Furthermore, it contains a controlling region (LCR) that determines whether the globin genes will be expressed during development and influences replication timing.

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Kitsberg *et al.* used two independent and rather elegant physical mapping techniques to locate an origin of bidirectional replication in the intergenic region between the δ and β genes, and exploited a small deletion of this region from an Hb Lepore thalassaemia patient to map genetically a DNA sequence necessary for replicator function to the same region. Strikingly, in the Hb Lepore cell line an *ori* was no longer located in the β -globin gene cluster and replication proceeded unidirectionally through the gene cluster,

presumably from another *ori* outside the locus.

This is a long-awaited combination of genetics and physical mapping to demonstrate a specific replicator sequence in mammalian cell chromosomes. It should foster further genetic definition of the replicator and discovery of its partner in replication, the initiator. □

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GREENLAND ICE SHEET

Two cores are better than one

G. S. Boulton

THE apparent duplication of effort and of cost involved in drilling two long ice cores within 28 kilometres of each other in the Greenland ice sheet has been strikingly vindicated, as Taylor *et al.*¹ and Grootes *et al.*² show on pages 549 and 552 of this issue. The cores are part of the current international programmes to drill through the great ice sheets of Antarctica and Greenland, which have produced remarkable advances in our understanding of the evolution of Earth's climate and environment.

The ice sheets have been pictured as layered accumulations of frozen atmosphere representing the past 500,000 years at the Vostok drilling site in Antarctica and 150,000 years at the GRIP (Greenland Ice-core Project) and GISP2 (Greenland Ice-Sheet Project) sites in southern Greenland. The isotopic composition of the ice tells us about the varying temperature on the ice-sheet surface at the time when the original snowfall took place; gases trapped as the snow turned to ice tell us about changing atmospheric chemistry and concentrations of greenhouse gases such as carbon dioxide and methane; and dust layers in the ice tell us about the storminess of the Earth and dustiness of the atmosphere.

One of the exciting new findings of GRIP³ has been the demonstration that the last glacial period was punctuated, between about 80,000 and 20,000 years before present, by a series of abrupt warm periods during which surface temperatures increased by about 5–8 °C for a few hundred years. Similar temperature excursions have now been deduced for the surface of the north Atlantic Ocean from the evidence of deep ocean cores⁴, possibly related to fluctuations in the extent of the polar atmospheric cell and the polar front, and terrestrial stratigraphies are also being re-examined for signs of these events.

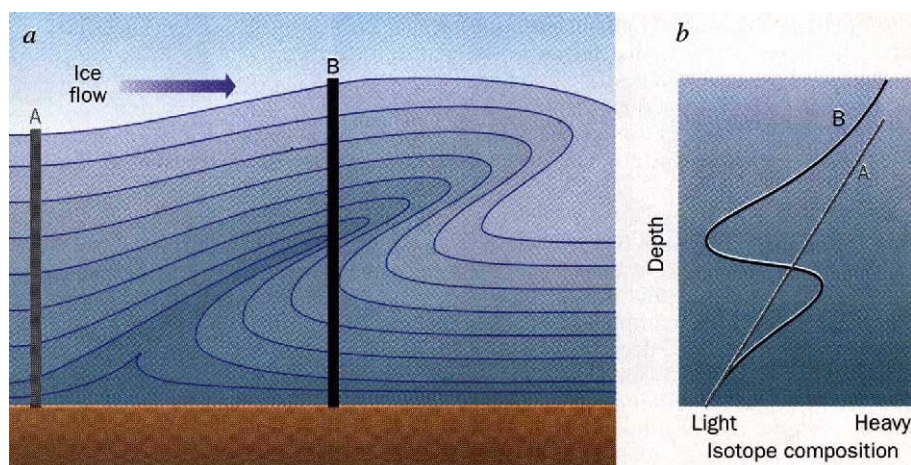
During the summer of 1993, further results from GRIP emerged that seemed⁵

to indicate a dramatic difference between the climate of the last interglacial period and our present climate. Whereas the present interglacial climate seems to have been very stable during the 10,000 years since the initial warming at the end of the last glacial period, the early and late parts of the last interglacial (135,000 to 115,000 years before present) showed rapid fluctuations between temperatures warmer

to yield extraordinarily rich climatic and stratigraphic information, some of us have become increasingly perturbed by the apparent absence of signs of flow deformation which we would expect in the basal ice because of flow over an irregular bed.

Now we may have found them. The comparison of the GRIP and GISP2 cores in this issue^{1,2} reveals the transition from a dominant stratigraphic signal in the upper part of the cores to one in which the stratigraphic signal has been strongly overprinted by flow effects. It confirms the reality of rapid temperature fluctuations within the last glacial period, but calls into question the inference of rapid climate fluctuations during the last interglacial. As an unexpected bonus, it has established an important deformational transition within the ice sheet.

Oxygen isotope records from the ice cores are presented in this issue by the GISP team² and electrical conductivity measurements by the GRIP team¹. High concentrations of alkaline dust are deposited on the ice sheet during cold periods, and this reduces the electrical conductivity of ice compared with that deposited in warm, less dusty periods. In both the oxygen-isotope and conductivity



a, A typical shear fold in the basal part of a glacier. If the ice had not been previously folded, a stratigraphic sequence of a climate-related property (such as oxygen isotope composition) sampled by a borehole at point A might produce a simple monotonic trend as shown by line A in part b. Sampling at B, after folding, would yield the sequence shown as B in b. Multiple folding can complicate the sequence further⁸.

and very much colder than the present. It apparently took only a decade or two to shift between these states. There was speculation that, as the last interglacial was warmer on average than ours, such climate instability might be in store in a modern world threatened by further greenhouse warming.

Whereas glaciologists interested chiefly in climate tend to think of ice sheets as static stratigraphic accumulations, others see them as great flowing tongues in which most of the action is near the base. As ice-core analysis has penetrated nearer to the ice-sheet bed, and as it has continued

records, there is a remarkable agreement between the cores from the surface to a depth representing an age of about 95,000 years before present (2,700 m in GISP2 and 2,670 m in GRIP). Below this depth, although there are some common general trends, there is little detailed correlation between them.

Why do the ice cores differ? An obvious explanation is that ice inhomogeneities and the irregularity of the bed generate more complex secondary stress fields near to the base of the ice sheet where strain rates are larger, thus tending to produce folds. Such folds intrude lower ice into

higher horizons of different composition (see figure), whilst multiple progressive folding can produce complex sequences such as are reflected in the spikes in electrical conductivity and oxygen isotopes which dominate the lower parts of the GISP2 and GRIP records.

However, GRIP is at the current ice divide where there is vertical compression and horizontal stretching, but no shear flow to generate folds. GRIP could therefore be a true stratigraphic record, apart from possible necking of some layers (boudinage) because of stretching, which could pinch out some stratigraphic horizons. The spikier record of GISP2, lying some 28 kilometres away on the flank, where ice flow is faster, could in contrast be the one strongly influenced by shear folding. On the other hand, it is likely that the ice divide has shifted in the past because of climate change⁶ and that a past shear-flow regime at GRIP has generated compositional spikes by folding. The test, of course, would be to drill another borehole on the divide.

Although observations at the base of the cores show relatively steeply inclined layers which are interpreted as folds, the attenuation of folds by flow after they have been first generated can produce a smoothly layered sequence in which fold terminations are rare. High inclinations are not necessarily a characteristic of folded sequences. We can safely assume, however, that folds have not been stretched over 28 kilometres to produce the identical sequences in the upper part of the cores.

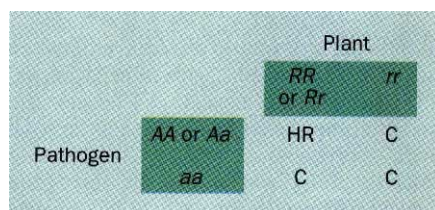
In a News and Views article in July⁷, Jim White drew attention to the remarkable memory of events in the last interglacial retained by the GRIP core. The GISP2 and GRIP comparisons reveal that this memory can be deceptive. Nonetheless, it may be that a detailed record can still be pieced together once we know more about the disruption to the deposited layers. The GRIP and GISP2 teams can now be expected to re-focus some of their efforts, from stratigraphy in the upper part of the core to structural geology at the base, not merely to disentangle the stratigraphic signal, but to take advantage of an excellent opportunity to understand deformation at the base of an ice sheet. □

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Debugging tomatoes

Robert Shields and Rebecca Stratford

MOST plants are resistant to most diseases — if they were not the landscape would be very different from what it is today. But although selection for disease resistance is central to much of crop improvement, little is known about how resistance genes work at a molecular level. Last year, a landmark was passed with the cloning of a maize gene whose product provides resistance to a fungal pathogen by degrading the fungal toxin¹. Here the mechanism of disease resistance is relatively straightforward. In a report in *Science*², Martin and colleagues now describe the cloning of



Gene-for-gene interactions between dominant plant resistance genes (*R*) and the complementary pathogen avirulence gene (*A*). The presence of both dominant alleles results in the hypersensitive resistance reaction (HR); all other combinations lead to plant infection and a compatible interaction (C).

another plant resistance gene. This time the plant concerned is tomato and the story is more intriguing.

Much work of plant molecular pathologists has centred on gene-for-gene resistance. If a plant resistance (*R*) gene 'recognizes' a pathogen's avirulence (*avr*) gene then the plant mounts a localized hypersensitive response: cells near the site of pathogen invasion rapidly produce low-molecular-weight, anti-microbial compounds (phytoalexins), followed by a number of so-called pathogenesis-related proteins (which have anti-fungal and other activities). Shortly afterwards the cells collapse to produce a desiccated necrotic lesion which contains the pathogen at the site of entry. Another cultivar of the same plant species lacking the *R* gene corresponding to the pathogen's *avr* gene will succumb to infection (see figure).

The model predicts that transferring the *avr* gene to a virulent pathogen will make it avirulent on plants carrying the corresponding resistance gene, and that mutation of the avirulence gene will result in virulence. Both predictions have been borne out^{3–5}, and the gene-for-gene model has been remarkably successful in describing the cultivar-specific interaction of viral, fungal and bacterial pathogens. It is hardly surprising, then, that a lot of effort has been put into cloning *avr* genes from

pathogens and their corresponding resistance genes from plants.

The simplicity of the gene-for-gene model led to the idea that there must be a direct interaction between resistance and avirulence gene products analogous to that between a receptor and its ligand. Indeed, in some cases, the protein encoded by avirulence genes directly elicits the hypersensitive response in plants with the corresponding resistance gene in the absence of the pathogen. For instance the coat protein of tobacco mosaic virus turns out to be an avirulence gene corresponding to *N* resistance gene in *Nicotiana sylvestris* (a relative of tobacco)⁶. Transgenic plants expressing the coat protein in every cell mount a systemic hypersensitive response if the host plant has the appropriate resistance gene⁷. Similarly, the peptide encoded by the *avr9* gene of the tomato leaf mould fungus, *Cladosporium fulvum*, causes rapid necrosis only when injected into leaves of tomato plants containing the *Cf9* resistance gene⁸. When the resistance genes corresponding to these avirulence gene products are cloned (and rumour has it that this is imminent), then it might be clearer if the resistance gene is some sort of receptor for the avirulence gene product.

Martin *et al.*² looked at the tomato *Pto* gene, which gives resistance to strains of *Pseudomonas syringae* pv. tomato (the causal agent of bacterial speck) carrying the previously cloned⁹ avirulence gene *avrPto*. The *Pto* gene was known to lie on chromosome 5 and in a *tour de force* of positional cloning Martin *et al.* identified at least six related expressed genes in the *Pto* locus. Susceptible tomato plants transformed with complementary DNA corresponding to a messenger RNA transcript from this region became resistant to *avrPto* carrying race 0 (but not other races) of bacterial speck, showing conclu-

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sively that the *Pto* resistance gene had been cloned. Far from being the expected receptor (with, for instance, a membrane-spanning domain) the protein encoded by the gene appears to be a serine-threonine protein kinase.

Preliminary results on the structure of the *Pto* locus suggest that intralocus recombination events may have occurred which may be a mechanism for the generation of diversity. This could explain why, in many plants, race-specific (and indeed other) resistance genes frequently map to the same position¹⁰.

So now both partners of a gene-for-gene interaction are known in some detail. How they interact is another question. On the pathogen side, the *avrPto* gene, in common with other avirulence genes from bacterial pathogens, has no homology to other proteins in the databases (so no

clues there as to how it might work)¹¹. One possibility is that *avrPto*, like the *avrD* gene (also from *P. syringae* pv. tomato), leads to the production of a low-molecular-weight elicitor of resistance¹². This could then interact with the *Pto* gene product, or at a point higher up the signal-transduction pathway, to produce a hypersensitive response. Like *avrD*, the *avrPto* gene expressed in the soybean pathogen *P. syringae* pv. *glycinea* elicits the hypersensitive response on soybean in a race-specific manner⁹. This leads to the prediction that the relevant soybean cultivar should contain a resistance gene similar to *Pto*. Likewise, an *Arabidopsis* resistance locus has been identified using avirulence genes from a soybean pathogen¹³. Avirulence genes from bacteria specific for different plants can be nearly identical (implying similarity of the

corresponding resistance genes), so it is possible that resistance genes cloned from one plant may function in another¹⁴.

Practical (as opposed to molecular) plant pathologists have limited use for gene-for-gene resistance as it is not always durable; moreover, most plant disease resistances do not seem to be of the gene-for-gene type. Where these molecular studies may prove more useful is in the elucidation of the signal-transduction pathway resulting in the hypersensitive response, and in the clues that this may give as to how to turn it on in response to any pathogen rather than those rarities carrying specific avirulence genes. □

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OBITUARY

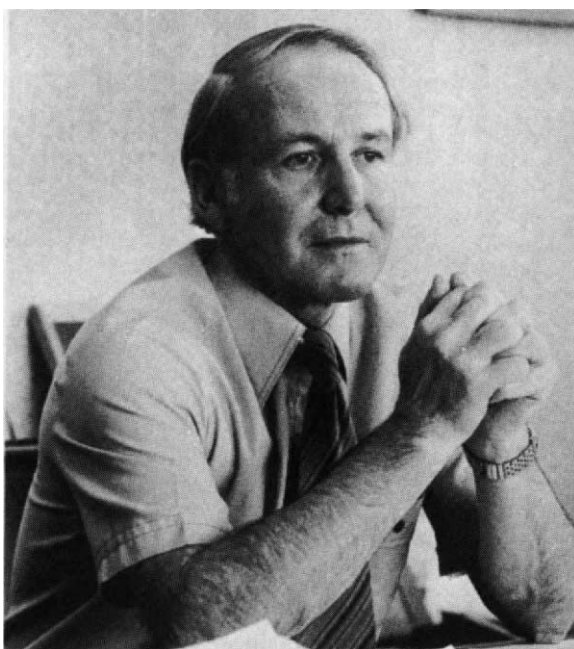
A. E. (Ted) Ringwood (1930–93)

"Our understanding of the Earth in all her aspects has developed dramatically in the last 25 years. This has been an exhilarating period to have been an Earth scientist. I feel very fortunate and fulfilled to have been able to participate in some of these developments."

These were Ted Ringwood's words in 1991 before the Italian National Academy as he accepted the Feltrinelli Prize, one of the most valued international prizes for scientific or cultural achievement. His premature death from cancer, on 12 November, has robbed Earth sciences of one of its most creative and stimulating contributors. Since the 1960s, Ted Ringwood's name has been synonymous with frontier research on processes in the Earth's upper mantle and the nature of the mantle transition zone. His pioneering studies at that time used germanates as analogues for high-pressure silicate phases, and he continued to lead this field, by a combination of prediction and experimental confirmation, for over 30 years.

Ted Ringwood was born and educated in Melbourne. His initial training in geology took a new direction when, for his Ph.D. studies at the University of Melbourne, he applied the geochemical concepts of V. M. Goldschmidt to the prediction of new mineral structures that should be stabilized by the very high pressures and temperatures of the Earth's mantle. After a brief post-doctoral position at Harvard, he was recruited by John Jaeger in 1958 to the newly established department of geology and geophysics at the Australian National University. Throughout his career, he worked to ensure that this institution became recognized internationally for research excellence. He began his ex-

perimental work in 1959 with diamond-anvil synthesis of high-pressure polymorphs. I was recruited by Jaeger in 1962 to work with him and our close association continued until 1976. Our early work was on the origin of basalts and their conversion to high-pressure eclogite (garnet and pyroxene). We explored the concept of the



A. E. Ringwood

gabbro-to-eclogite transformation acting as a tectonic engine within both a model of continental growth and a 'sea-floor spreading' model of ridges and trenches. Later, Ringwood was to develop the idea of how phase transformations in the subducted slab controlled mantle convection. His strength lay in seeking points at which models could be tested — either by real Earth data or by an experimental approach. Always forceful and often controversial in

debate, he challenged interpretations from geophysical observations when they conflicted with geochemical data or models.

Although primarily directed at understanding the deep Earth, his very-high-pressure studies also took him into the design and demonstration of ultra-hard materials based on diamond aggregates and synthetic cubic boron nitride. He also turned his knowledge of mineral chemistry to an issue of global concern — the safe disposal of high-level nuclear waste. He devised and demonstrated the 'SYNROC' concept of containing radioactive elements in stable mineral assemblages. He saw clearly that excellence in basic research was necessary to solve practical problems, but also worked to ensure that potential applications of his basic research were followed through.

Ted Ringwood was a vigorous contributor to debates on the evolution of the Solar System. With the return of the lunar samples, he used the experimental approach to investigate the origin and significance of lunar basalts. As ever, his fertile mind led him to present speculative hypotheses, rapidly followed up with experimental tests. He could look back over a career studded with ideas, some now discarded, others

forming the firm foundation of current knowledge. Ted Ringwood's death at a time of continuing productive research is a great loss — he was one of the most internationally honoured of Earth scientists and a great Australian. David Green

David Green will be Director of the Research School of Earth Sciences at the Australian National University, Canberra, Australian Capital Territory 0200, Australia from January 1994.

RÉSUMÉ

No neck

THE myosin head is divided into two domains, one containing the ATPase and binding sites, the other (the neck that connects the head to the filament trunk) consisting of a long α -helix to which the two light chains are attached. This neck is thought to convert the movement of the jaws around the ATPase site into the power stroke that drives the head along the actin filament. But things may not be so simple. S. Itakura *et al.* (*Biochem. biophys. Res. Commun.* **196**, 1504–1510; 1993) have prepared a neckless myosin head, and coupled a reactive cysteine to its neck end, as well as to wild-type heads; this allows the heads to be attached to a surface in a defined orientation, by way of an avidin–biotin link. The surprise is that the mutant heads apparently propel actin filaments along almost as well as the wild type.

Bang on course

ASTRONOMERS and others with a relish for celestial fireworks will be pleased to hear that Shoemaker–Levy 9, known as the 'string of pearls' comet, has definitely made a date with Jupiter. The latest accurate orbital parameters have been computed by J. V. Scotti and T. Metcalfe for nine of the individual cometary nuclei, from images obtained with the Spacewatch telescope between March and July this year (before the comet disappeared behind the Sun), and are published in *Minor Planet Circulars* of 29 November. Armed with advance notice of these computations, B. G. Marsden (*IAU Circ. No. 5893* (22 November 1993) has pinned down the expected collision times to within a couple of hours: nucleus 17 will hit the far side of the planet at 1994 July 18.7 (Universal Time); nucleus 15 at July 19.1; 14 at 19.6; 12 at 20.2; 11 at 20.9; 7 at 21.6; 6 at 22.1; 5 at 22.7; and finally, to round off five remarkable days in Jupiter's recent history, nucleus 1 is due to plummet into the planet at July 23.2.

Small advantage

VOLES are not ruminants. So why is their digestive efficiency so much higher than, say, that of cows? The answer, according to W. B. Lee and D. C. Houston, lies in their ability to chew their food — often plant leaf material — into very small particles, so giving their digestive enzymes a head start. As part of a wider study (*J. Zool.* **231**, 301–309; 1993), Lee and Houston put voles on high-fibre and low-fibre diets, and examined the resulting size of food particles; they also looked at tooth microstructure. Voles can grind exceedingly fine, seemingly a consequence of their tooth design, and tooth wear alters with diet. This, the authors speculate, may result in maintenance of high chewing — and digestive — efficiency as food quality changes.

The binding issue

D. Colquhoun and M. Farrant

ON page 565 of this issue¹, Amin and Weiss show that certain mutations in the amino-terminal region of a GABA_A-receptor subunit reduce the effectiveness (potency) of the agonist, GABA (γ -aminobutyric acid, the major inhibitory neurotransmitter in the brain). But is it the ability of GABA to bind that is reduced, or the ability of GABA to open the channel once bound? If it is the former, then the mutated amino acids may be part of the agonist binding site, so the answer to this question is important. Amin and Weiss have addressed the problem clearly, and give reasons for thinking that it is mainly affinity that is reduced. The distinction is not an easy one, however, and has often been misunderstood. We are now seeing the painful rediscovery by molecular biologists of a standard bit of 1950s pharmacology.

The unthinking reaction to the problem is "If you want to know whether binding affinity is reduced then do a binding experiment". But consideration of even the simplest mechanism for agonist action (see, for example, ref. 2) shows that the binding of an agonist, as measured in a binding experiment, reflects not only its ability to bind initially to the receptor, but also its ability to open the channel once it is bound. The second step, the isomerization between shut and open states both of which have agonist bound, is referred to as 'gating' in the ion-channel literature. This characteristic of binding measurements is expected generally, because of the physical principle of reciprocity (see, for instance, ref. 3): *binding affects gating so gating will affect binding*.

Distinguishing between the effects of mutation on gating and on binding is exactly the same problem as that which led Stephenson⁴, in 1956, to distinguish between effects on agonist efficacy and effects on affinity; he, of course, was thinking of effects of changes in agonist structure rather than effects of changes in receptor structure, but the principles are the same. Stephenson showed that if the agonist has high efficacy (that is, in the present context, more than 95 per cent or so of channels are openable by high agonist concentration), changes in affinity were quite indistinguishable from changes in efficacy (gating) on the basis of equilibrium concentration–response curves (increasing either causes a parallel shift to the left with a log concentration scale).

It is because of these problems that most investigations of structure–function relationships in ion channels (such as the now classical study on the effects of rings of charges on ion permeation⁵) have, wisely, focused on effects of mutations on

the characteristics of the channel while it is open, which are easier to interpret than effects on binding and gating.

Although these principles have been established since 1956, the history of attempts to distinguish experimentally between affinity and efficacy has been tortuous. The classical methods are unlikely to work, because they too neglect the principle of reciprocity⁶. At this point, the patch clamp comes to the rescue. The information that can be obtained from single-channel recordings allows far more detailed investigations of mechanism than is possible in other systems such as enzymes. This gives ion channels a real advantage in investigations of the structure–activity relationships of proteins (though it is not yet possible to obtain detailed three-dimensional structures of normal and mutated ion channels, which can be done with enzymes).

Measurements of the fine structure of channel openings can, in favourable cases, allow separation of affinity and efficacy (see ref. 7). It is this approach that Amin and Weiss have used. They suggest, on the basis of single-channel data yet to be published, that GABA is an agonist of relatively low efficacy; the open–shut equilibrium constant is estimated to be around 4 — that is, only 80 per cent of channels are openable (in contrast, the equilibrium constant of the nicotinic acetylcholine receptor is around 40, so 98 per cent of channels are openable). If they are right then, in this case, any substantial effect of the mutations on gating would have resulted in a reduction of the maximum response, which was not observed (maxima are of course not easy to estimate precisely, because of problems such as desensitization, but that is another question). The authors therefore conclude that the mutations have probably reduced the initial binding of GABA, and that therefore, with a bit of luck anyway, the mutated residues may have been in the agonist binding regions of the protein.

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If these arguments are correct, the amino acids that are crucial to agonist binding are in the β -subunit. Amin and Weiss find two regions of the $\beta 2$ subunit, separated by 41 amino acids, with sequences $Y_{157}GYT$ and $T_{202}GSY$, which each appear to contribute a crucial tyrosine and threonine (indicated in bold). Conservative mutation of any of these four amino acids produced a large reduction in GABA potency, whereas mutations of several other amino acids in the region (or in the analogous regions of the $\alpha 1$ and $\gamma 2$ subunits) had relatively small effects. It is also interesting that mutations of the critical amino acids had little effect on activation of the channel by the barbiturate pentobarbitone (also known as pentobarbital): this elegantly confirms the view that GABA and pentobarbitone act on different sites.

The new findings do not exclude the possibility that GABA may bind to subunits other than the β (see, for instance, ref. 8). Indeed, expression of α , γ , δ or ρ

subunits alone, or in heteromeric combinations that lack a β -subunit, can produce functional GABA-activated channels, albeit with some variability⁹⁻¹¹. Each of these subunit types contains a tyrosine residue homologous with βY_{157} , and, with the possible exception of the δ subunit, at least some features of the two binding domains reported by Amin and Weiss. Whether these homologous regions in other subunits can also bind the agonist remains to be seen.

As more and more studies on mutated receptors appear, the question of whether or not mutations affect the initial binding of agonists will crop up again and again. Amin and Weiss have made a better attempt at answering it than most. □

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CRETACEOUS/TERTIARY BOUNDARY

Extinctions at the antipodes

Kirk R. Johnson

No one should expect that the second largest extinction in Earth history would turn out to be a simple affair. Although the theory that a bolide impact caused the mass extinctions at the Cretaceous/Tertiary (K/T) boundary has received wide acceptance in the 13 years since its proposal, most of the supporting data have come from mid-latitude sites, where there is evidence of the impact debris and its source crater, and detailed biostratigraphic records of large, abrupt extinctions. In contrast, the message emerging at a conference in Boston, Massachusetts*, was that the effects of the K/T event on life near the ancient South Pole were not particularly severe and, in some cases, were hardly noticeable. In addition, there is increasing evidence from a range of palaeolatitudes that large-scale plant, mollusc and nannofossil extinctions preceded the K/T boundary event by several million years.

When the impact-extinction hypothesis was first proposed, it appeared to be easily testable. In its simplest form, the hypothesis predicted stratigraphically abrupt extinctions, followed by low-diversity assemblages of surviving species. The K/T boundary record at high palaeolatitudes adds a complication to this prediction, that of the odd structure of extinct polar ecosystems. It is now generally accepted that Late Cretaceous polar regions,

although subject to intensely seasonal regimes of light and dark, were nonetheless ice-free and forested¹. There are no modern analogues for these ecosystems, and their response to an abrupt catastrophe such as a bolide impact would have been strongly affected by the time of year at which it happened. Organisms living at high latitudes in the Northern and Southern Hemisphere would have had different responses because the event would have occurred in opposite seasons.

A further snag with high-latitude K/T

sections is that the distance from well-studied mid-latitude sections makes biostratigraphic correlation tenuous. One piece of good news at the conference was that the discovery of a significant iridium anomaly (R. Askin, University of California, Riverside) in fossil-rich sections on Seymour Island near the Antarctic Peninsula allows this site to be correlated to the K/T event. Among fossil dinoflagellates in this nearshore marine sequence, there is a decrease in the relative abundance of the dominant species above the iridium-bearing level, but there are only minor extinctions. The terrestrial pollen and spore record from this same section marks the boundary with the disappearance of only a few, rare taxa². To be sure, caution should be exercised in the interpretation of terrestrial palynomorphs in marine sediments, but a similar pattern of low palynofloral extinction has been observed at the K/T boundary in terrestrial deposits in New Zealand³. This contrasts strongly with the pattern in the mid-latitude Northern Hemisphere (D. Nichols, United States Geological Survey) of high palynofloral extinction, where loss of roughly 30 per cent of species coincides with the iridium anomaly.

At the other end of the globe, a stratigraphic sequence from Alaska's North Slope (L. Marincovich, United States Geological Survey) shows apparent survival of some Cretaceous molluscs (not including ammonites or inoceramids) into the Palaeocene. But this pattern may have as much to do with the disputed position of the K/T boundary in the Colville River section as with mollusc survival⁴. In the pollen record for this sequence, the boundary is defined by high levels of extinction in the *Wodehouseia spinata* assemblage and occurs above the level of the putative Palaeocene molluscs⁴.

The indisputably Cretaceous portion of

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

*The Cretaceous/Tertiary Boundary Event: Biotic and Environmental Changes, Geological Society of America National Meeting, Boston, Massachusetts, 27 October 1993.

Survivors — fossil leaves from the Lauraceae family, found in Nelson, New Zealand, represent one species that survived the K/T boundary event.

this section holds the remains of dinosaurs⁵, as do Early Cretaceous sites from high-palaeolatitude Australia⁶, indicating that dinosaurs were able to survive in polar light regimes. This has been offered as evidence that the impact-induced darkness and cold would not necessarily have killed off the dinosaurs^{5,6}, but such hypotheses fail to account for the extreme seasonality of ancient polar climates. The dinosaurs could survive a polar winter that followed a highly productive polar summer. Remove the summer light with bolide dust and follow it with another dark winter, and things look considerably tougher.

Does the observed pattern of high K/T extinction in the Northern Hemisphere and low K/T extinction in the high latitudes of the Southern Hemisphere suggest that the Chicxulub bolide arrived in the boreal spring? Or is the geographical pattern of extinctions still too poorly known for us to speculate? These questions will only be answered by a greater geographical diversity of high-resolution K/T boundary studies. At the conference, alongside the suggestions of unexpected survivals ran a parallel thread of evidence for substantial extinctions well before the K/T event. Belemnites and inoceramid bivalves from the Seymour Island section disappeared near the Campanian/Maastrichtian boundary, several million years earlier (W. Zinsmeister, Purdue University). Ammonites from this section, once thought to extend into the Palaeocene⁷, are not found *in situ* above the K/T iridium anomaly but do appear to decline in diversity towards the end of the Cretaceous period. At low palaeolatitudes, reef-building rudist bivalves disappeared about 1.5 million years before the boundary (C. Johnson, Pennsylvania State University).

Add these observations to other recently discovered events during the mid-Maastrichtian age, such as the major floral extinctions on land⁸ and inoceramid⁹ and nanofossil¹⁰ extinctions at sea, and it looks as though a bolide impact alone cannot explain the whole of the biotic crisis at the end of the Cretaceous. □

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The elusive signature of CH₅⁺

Gustavo E. Scuseria

Do all molecules possess a definite equilibrium geometry? Although many molecules exist in multiple isomeric forms, usually a structure can be identified as the lowest in energy and assigned as the ground-state geometry conformation. But, in a paper entitled "CH₅⁺: The never-ending story or the final word?", Schreiner *et al.*¹ conclude that protonated methane may be an exception to the rule.

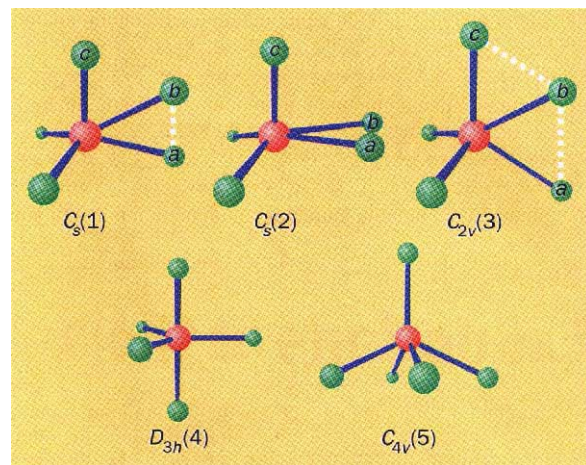
Known since the 1950s and now a common gas-phase reagent for chemical ionization mass spectrometry, protonated methane's small size makes very-high-level quantum mechanical calculations computationally feasible, and its penta-coordinated carbon atom gives a special importance to establishing its structure. CH₅⁺ is the smallest member and perhaps the prototype of the class of protonated alkanes or nonclassical carbonium ions, which play important roles as intermediates in acid-catalysed transformations of hydrocarbons and in many electrophilic reactions². Yet despite the interest in protonated methane, a high-resolution infrared spectrum remains elusive. Attempts made by top practitioners such as Saykally (University of California, Berkeley) and Oka (University of Chicago) have so far been unsuccessful.

The conventional view of the structure of CH₅⁺, shown as C_s(1) in the figure, corresponds to a CH₃⁺ unit strongly bound to a H₂ molecule by an energy of about 40 kcal mol⁻¹. This picture, supported by recent theoretical studies^{3,4}, is consistent with that of the nonclassical carbonium ions, which are known for forming three-centre/two-electron bonds⁵. Other isomers, such as C_s(2), C_{2v}(3), D_{3h}(4) and C_{4v}(5), have also been thought plausible candidates for the lowest-energy conformation. Previous calculations indicate that the C_{4v}(5) structure is higher in energy than C_s(1) by about 1 kcal mol⁻¹, and that the trigonal bipyramid D_{3h}(4) isomer is unfavourable.

In the latest twist to the CH₅⁺ story, Schreiner and co-workers have made high-level calculations demonstrating that although the C_s(1) structure is the best candidate for the global energy minimum in the potential energy surface, the C_s(2) and C_{2v}(3) structures are transition states

for hydrogen scrambling with remarkably small activation barriers. From calculations using large basis sets in conjunction with the coupled-cluster method^{6,7}, they conclude that the C_s(2) and C_{2v}(3) structures lie above C_s(1) by 0.1 and 0.9 kcal mol⁻¹, respectively. If zero-point vibrational energy corrections are included, the energy differences among these three structures become essentially negligible.

These state-of-the art theoretical pre-



Five possible structures for CH₅⁺. (1) Lowest-energy minimum in the CH₅⁺ potential energy surface. This structure corresponds to a hydrogen molecule (H_aH_b) strongly bound to a CH₃⁺ unit. (2) Transition state for rotating H_a-H_b about the CH₃⁺ unit leading to exchange of H_a with H_b. (3) Transition state for hydrogen scrambling that switches H_b and H_c, resulting in a H_bH_c molecule attached to CH₃⁺. Because (2) and (3) are low-lying in the potential energy surface, all five C-H bonds are essentially equivalent and exchange dynamically very rapidly. (4), (5) Higher-energy isomers of CH₅⁺.

dictions, based on what is widely accepted as one of the most accurate *ab initio* computational quantum chemistry methods^{8,9}, are significant because they clearly indicate that for all practical purposes CH₅⁺ does not have a unique, stable equilibrium structure. The hydrogen atoms are predicted to scramble almost freely among multiple equivalent minima. This effect, usually known as 'pseudorotation', yields very complicated spectra, which could be a true nightmare for experimentalists and may be already responsible for the spectroscopic elusiveness of CH₅⁺. If the theoretical predictions turn out to be correct, attempts to interpret the experimental data based on a single structure may be doomed to fail.

Just a few months ago, Boo and Lee¹⁰ attempted to stabilize CH₅⁺ by attaching to it a weakly bound neutral species such as H₂. In this experiment, the idea is to assess the structural features of CH₅⁺ indirectly by 'freezing' it with another small molecule which ideally stops the hydrogen

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scrambling while causing only a minor perturbation in the structure. The result (although low-resolution) was the first infrared spectrum of CH_5^+ . But theoretical calculations¹¹ have already indicated that although their $\text{CH}_5^+(\text{H}_2)$ complex is weakly bound as expected, it still has unrestricted internal degrees of freedom: multiple structures with H_2 bound to one of the protons of the three-centre/two-electron bond are predicted to have essentially the same energy, allowing again practically unrestricted rotational and scrambling motion of the H_2 about the CH_5^+ . So extracting precise structural information for the core CH_5^+ unit from this $\text{CH}_5^+(\text{H}_2)$ complex could be extremely difficult.

It seems, then, that the concept of a molecular 'equilibrium geometry' is not valid for CH_5^+ . Rather than a picture of a single structure about which the atoms vibrate, a model in which 'delocalized' vibrational modes allow atoms to move almost freely over large regions of space is probably more appropriate. The repre-

sentation of CH_5^+ stemming from the calculations of Schreiner *et al.* is that of a floppy molecule without a definite structure. Far from being the prototypical nonclassical carbocation, the authors conclude, CH_5^+ is unique. □

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ANCIENT DNA

Less cause for grave concern

Bryan Sykes

IT'S been a busy time for those involved in analysing ancient DNA since That Film came out this summer. Having spent a few months reassuring the public that cloning dinosaurs, as portrayed in *Jurassic Park*, is science fiction, they met in Washington* to reassure each other that at least the survival of DNA beyond the grave was anything but.

Gone, thank goodness, are the days when merely obtaining an amplification product from some biological antique was grounds for celebration, especially when its sequence vaguely resembled something in the nucleotide databases. In the two years since the first international conference in Nottingham¹, when everyone in the field woke up to the unpleasant fact that contamination with modern DNA was all too common, the mood has become one of caution rather than exuberance. All of the speakers in Washington stressed the need for stringent precautions for avoiding contamination, including the vigorous surface treatments of specimens to remove DNA-rich fingerprints.

This is, of course, a particular problem when dealing with human bones, but there are several examples of apparently authentic recoveries of human DNA that have survived rigorous decontaminating procedures. Among these are an impressive series of 98 skeletons excavated from a 2,000-year-old Amerind site in Norris Farms, Illinois (A. Stone, Penn State

University), a smaller series from an older palaeoindian site at Port au Choix, Newfoundland (B.S.), several Central and South American mummies (A. Merriwether, University of Pittsburgh; C. Kolman, Smithsonian Tropical Research Institution) and remains from Easter Island (E. Hagelberg, University of Cambridge). These are all amplifications of mitochondrial DNA which, despite some widely publicized statistical setbacks (see, for example, ref. 2), continues to be a most informative marker for at least relatively recent human population movements.

Accepting these derived sequences as genuine is easier because they all fit into an appropriate phylogenetic context; indeed, the construction of these contexts using DNA from extant populations is proving to be absolutely vital. Another good sign that sequences are genuine is a concordance between anthropometric and DNA-based sexing, which was achieved in both Norris Farms and Port au Choix populations using either Y-chromosome-specific repeats or sex-chromosome-specific differences in the amylogenin gene.

Contamination neurosis is understandably less severe among those who work on animal and plant remains, and there were convincing accounts of credible sequences from, among others, the giant ground sloth (M. Höss, University of Munich), extinct cave bears (C. Hanni, Institut Pasteur de Lille), long-dead rabbits

(M. Monnerot, CNRS, Paris) and extinct Hawaiian birds (A. Cooper, Smithsonian Institution). The over-enthusiasm of nineteenth-century collectors has been put to good use when comparing the genetic diversity in museum collections with that of extant populations which, like the whooping crane (T. Quinn, Smithsonian Institution) and the red panda (R. Wayne, Zoology Society of London), have gone through a population bottleneck in recent times.

Phylogenetic fidelity, however, is the least of the worries as far as the amazing claims that DNA survives in amber for over 100 million years are concerned. Both G. Poinar (University of California, Berkeley) and R. Cano (California Polytechnic, San Luis Obispo) catalogued the painstaking precautions they had taken during extraction of DNA from entombed weevils³ and bees⁴. Nothing obvious had been overlooked, and the sequences of both the insects and their indigenous enterobacteria (interestingly confined to the abdomen) are plausibly different from their modern equivalents.

The opposition was in the form of the spiritual (but not physical) presence of T. Lindahl, whose disbelief that DNA can survive time-dependent, mainly oxidative, decay much beyond 50,000 years stems from extrapolations of experimentally determined rates of damage in solution^{5–7}. This clash between pride and prejudice will not be resolved until there is ample independent verification of the amber data. On a hopeful note, it seems that the damaged DNA from ancient specimens is not beyond repair, using glycosylases to sand down the DNA, then polymerases and ligases to fill and polish it (L. Grossman, Johns Hopkins University); and there has been at least one successful attempt at reviving Pleistocene DNA using the repair systems of *Escherichia coli* (Höss).

The power of ancient DNA is at its greatest when it can answer historical questions posed by other disciplines, such as the colonization of the Americas and the Pacific and, in Europe, the Anglo-Saxon settlement of England. After the shock of Nottingham enough careful, rigorous and reproducible work has been done to at least reassure those present that this really is going to work and that we are not, after all, dealing with the molecular biological equivalent of cold fusion. □

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Ruthenium route to reaction

Alan S. Goldman

CARBON–hydrogen bonds are ubiquitous in organic chemistry. Yet although the organic chemist has a wide repertoire of reactions that are highly specific for transformations of various functional groups, the ability to react sp^2 - and sp^3 -hybridized C–H bonds is limited. There are virtually no reagents that can choose between similar C–H bonds in a complex organic molecule and add a functional group to a specific site; developing catalysts for this is one of the great challenges of modern chemistry. A big step towards this goal is described on page 529 of this issue by Murai *et al.*¹, who have found ruthenium complexes that can efficiently and selec-

tively catalyse the insertion of olefins into specific C–H bonds of arylketones.

Organometallic chemistry, which has given rise to many of the most useful and specific catalysts for organic reactions, has long been considered promising for catalytic functionalization of C–H bonds. Numerous reactions of organotransition-metal complexes with C–H bonds have been discovered in the past two decades². Many of these reactions have attractive selectivity patterns; for example, several examples of specific attack on the terminal position of *n*-alkanes have been found³. But incorporating such reactions into catalytic cycles has proved difficult; although

some catalytic examples have been discovered (for example, alkane dehydrogenation⁴, as in equation (1), and the insertion of CO (equation (2), ref. 5) or isocyanides⁶ into C–H bonds), selectivity and yields have generally been much too low for organic synthesis⁷.

As organometallic catalysts are widely used to effect the addition of H_2 (H–H bonds) to olefins, a logical step was to try the analogous insertion of olefins into C–H bonds. Unlike the reactions in refs 4–6, olefin insertion is thermodynamically very favourable. In 1989 Jordan and Taylor discovered a cationic zirconium complex that could catalyse the insertion of ethylene, propene or 1-butene into the *ortho* C–H bond of picoline⁸, as shown in equation (3).

The system that Murai and co-workers now report, although related, seems to be much more general. It may prove to be the first synthetically useful example of an organometallic-catalysed transformation of a C–H bond. $H_2Ru(CO)_2L_2$ and $Ru(CO)_2L_3$ (where L is $P(C_6H_5)_3$) are found to catalyse the reaction of a huge variety of aromatic ketones with a wide range of olefins, according to equation (4).

The reactions are highly efficient. Up to 50 mol of product is obtained per mol

of ruthenium. More important, in many cases quantitative yields of product are obtained based on both olefin and aromatic ketone. In all previous examples of the catalytic functionalization of unactivated C–H bonds, a large excess of at least one reagent has been required; in most cases the substrate containing the C–H bond has been used as the solvent.

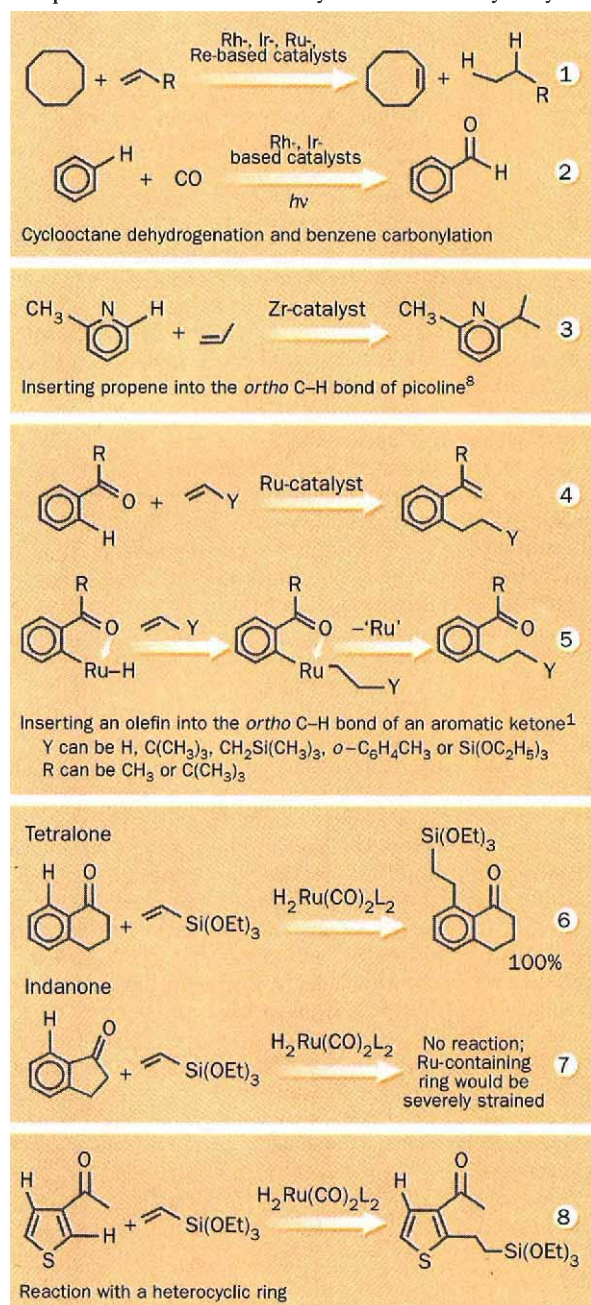
Substitution reactions specific to the *ortho* position are unusual. Electronic effects that favour *ortho* rather than *meta* attack inevitably also favour attack at the *para* position, and steric effects generally favour *para* attack over *ortho*. The key to the site-specificity of the reaction demonstrated by Murai *et al.* presumably involves coordination of the ketone carbonyl group, positioning the ruthenium to react with the *ortho* C–H bond. The olefin then inserts into either the Ru–aryl or the Ru–H bond (shown), and the product is eliminated (equation (5)).

The carbonyl-bound ruthenium–aryl complex is strongly implicated by a comparison of the reactivity of tetralone (equation (6); 100 per cent yield) with indanone (equation (7); no reaction). In the case of indanone the ruthenium-containing ring would be highly strained.

For synthesis, another important aspect of the reaction's high selectivity is the direction of addition. The aryl group is added to the less hindered side of the olefin. This is presumably because of steric effects that keep the Y group away from the crowded ruthenium centre during the olefin insertion. Additionally, and rather surprisingly, where two inequivalent *ortho* C–H bonds are present, Murai and colleagues find that selectivity is virtually complete for one of the two (equation (8)).

The next question, of course, is how general these reactions are. What other functional groups (on either substrate) can be tolerated? What other types of olefin can be inserted into the C–H bond? The answers will largely determine the range of applications that the reaction will find in organic synthesis. More broadly, for either this ruthenium catalyst or other organometallic complexes, it remains to be seen what other functional groups will act to 'direct' the functionalization of specific C–H bonds. □

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For richer, for poorer

Peter D. Moore

Is size a matter of importance among seeds? Maranon and Grubb¹ have looked into the question as it concerns Mediterranean annuals and have come up with an answer — their conclusion is that big seeds equip a plant for growth in rich environments.

Seed size is normally a remarkably consistent character within any given species of plant, meaning that this feature has probably evolved in response to strong and persistent environmental pressures. Yet the precise value and ecological advantage of a particular size of seed, be it large or small, is still a matter for conjecture. It is not even clear whether large-seeded species are better equipped for low- or high-resource habitats.

A wide range of factors needs to be taken into consideration when assessing the potential advantages of producing large seeds. Dispersal by wind is hampered, but dispersal by animals or water may not be (consider the coconut). Resources for seedling establishment are in rich supply, which could be advantageous if the plant has to cope with competition from other plants or with a lack of such requirements as water or minerals in the early stages of growth. If long-term dormancy is likely, a high seed weight is apparently distinctly disadvantageous, because most long-dormant weed seeds (such as those of poppies) are small. But protection from fire or insect predation could demand a thick seed coat. Put all this together, and it becomes evident why general principles relating to the adaptive value of particular seed sizes have been hard to find^{2,3}.

With respect to the resource richness or poverty of the site in which germination and establishment are to take place, however, one might predict that a large-seeded species is at an advantage under conditions of low resource availability. In the fynbos heathlands of southern Africa⁴, for example, members of the Proteaceae bear few, large seeds, as they do in Australia where as much as 40 per cent of the annual uptake of phosphorus by the parental plant may be invested in seed production⁵. Presumably this high investment equips the seed with a valuable store of this vital element as it germinates and establishes itself in the phosphorus-poor soils of these areas.

On the other hand, it could be argued that environments rich in resources such as light, water and minerals are likely to be inhabited by robust, fast-growing, competitive species⁶. Under such conditions the extra start in life offered to a seedling by nutrient supplies in the seed could make a substantial difference to its estab-

lishment. But this view assumes that growth is faster in the seedlings that develop from big seeds. In fact, studies of various plant families have demonstrated that the reverse may be true, and that the relative growth rate (RGR, the rate of weight increase per unit of initial weight) has a negative relationship to seed weight. Maranon and Grubb have examined 27 species of annual Mediterranean plants, including grasses, composites and legumes, and have confirmed that, in these species, high RGR is associated with small seeds.

Plant growth can be considered in terms of two components — the unit leaf rate (ULR, the rate of weight increase per unit area of leaf), which is an expression of the photosynthetic efficiency of leaves; and specific leaf area (SLA, leaf area per unit weight of plant). When the two were examined separately in the test species, Maranon and Grubb found that ULR was greater in the large-seeded species, but that SLA was less. So the low RGR of large-seeded plants is a consequence of their poor rate of production of leaf tissue per unit initial weight. What seems to be happening is that the large seeds are producing thick leaves that are efficient at using light (hence their high ULR), but their rate of leaf production is relatively inefficient considering their initial weight advantages. Overall, small-seeded species are able to intercept more light per unit mass.

Relative growth rates can be misleading, however. Larger seeds do give rise to large seedlings but, on the basis of the observations described here, the greater RGR of the small-seeded species would

allow them to overtake their advantaged colleagues within six weeks, as long as competitive interactions had not interfered with the process. Understanding the precise effect of competition demands a knowledge of density as well as growth analysis, so it is a complication that Maranon and Grubb have not fully faced. They have, however, gone back to the field to check where the small- and large-seeded plant species mainly occur. In the richer, deeper soils with higher organic matter derived from tree leaf-litter, large-seeded species, such as the grass *Bromus diandrus* and the thistle *Carduus pycnocephalus*, were typical. In the shallow, resource-poor sites, small-seeded species, such as the smooth catsear *Hypochoeris glabra* and the grass *Agrostis pourretii*, predominated.

Maranon and Grubb believe that, as far as Mediterranean habitats are concerned, they have hit upon a reasonably robust generalization. Large-seeded plants are at their best in environments with rich resources and small-seeded ones have an advantage in poverty-stricken sites. It remains to be seen whether the generalization will hold good in other geographical areas and other ecological situations, when, for example, the limiting resource is not water or minerals but light. □

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GREENHOUSE GASES

The mutable carbon sink

John Taylor

OUR inability to balance the global carbon cycle has led to the widespread use of the term 'missing sink'¹ or 'missing carbon' to reflect the fact that our estimates of the sources of carbon dioxide to the atmosphere exceed our estimates of the sinks by about 2 gigatonnes of carbon per year. The latest review of the uptake of CO₂ by the oceans², as derived from ocean modelling studies, concluded that it is the terrestrial biosphere which is taking up the excess carbon. The challenge is to identify the location of this sink and describe the processes by which it is operating.

Two papers^{3,4} in *Global Biogeochemical Cycles* consider this question and arrive

at very different conclusions. Richard Houghton³ finds that data available from forest surveys do not support the hypothesis that the temperate regions of the Northern Hemisphere are a significant sink for atmospheric CO₂. Aiguo Dai and Inez Fung⁴, in contrast, report that climate changes over the past 60 years have produced a CO₂ sink of about half of what is required to balance the carbon budget, and that this sink does lie predominantly in mid-latitudes in the Northern Hemisphere.

Houghton³, in a paper that seems sure to be controversial, argues that the search for the missing carbon may be "getting out

of hand"; many hypotheses exist, but none has yet been demonstrated to apply globally. This view is not surprising given the complexity of the carbon cycle, the urgent need to improve our understanding of the cycle and its role in climate change, and the absence of adequate constraints. Then, too, there is the political angle — you might want to 'discover' a substantial CO₂ sink in your region if this can be used to offset industrial emissions of CO₂; on the other hand, this may bring with it restrictions on land use with concomitant economic penalties.

Two earlier analyses of forest growth^{5,6} produced high estimates of CO₂ uptake in the temperate and boreal regions of the Northern Hemisphere, and at first sight might seem to have identified these forested regions as the missing sink. Houghton takes issue with these findings, on the basis that they did not properly account for the fate of carbon 'harvested' from the forests — they took account of carbon-consuming regrowth after logging, which has increased substantially in the past 25 years, but not the CO₂ released from decay of plant material, whether it rots as debris on the forest floor or gradually oxidizes in final, processed form (say, lumber or paper). In fact, Houghton suspects that the net flux of carbon from such changes in land use may be close to zero. Detailed, mass-conserving biogeochemical models that track carbon from its uptake by the biosphere through its storage and return to the atmosphere will be needed for reliable estimates of the net flux of carbon to the atmosphere.

Houghton also argues that if new plant growth really accounts for a carbon imbalance of 1.5 Gt C per year, this growth rate should be detectable from forest surveys, whether it occurs in the northern temperate zones alone or around the world. Available forest surveys should be scrutinized; but questions about how accurate and representative they are make it unlikely that these surveys alone will give the definitive answer on the size of the forest carbon sink.

Dai and Fung⁴, using empirical models for net primary production and soil respiration in conjunction with global temperature and precipitation data, have investigated the effects of climate change on CO₂ uptake for the period 1940–88, finding that variations in temperature and precipitation could have produced a cumulative carbon sink of 20±5 Gt C for the period 1950–84. They ignored the effect of increases in plant growth associated with increasing atmospheric CO₂, the 'CO₂ fertilization effect'.

If Dai and Fung are correct in their assessment of the importance of climate change for the biospheric uptake of CO₂, then understanding this mechanism will be vital for accurate prediction of future CO₂ concentrations. But as they acknow-

ledge, they used a greatly simplified empirical model to calculate the flux of carbon between the biosphere and the atmosphere that only takes into account changes in temperature and precipitation. It does not account for the dynamics of carbon storage, thereby linking net primary productivity (NPP) to respiration, or other factors limiting NPP. They used a modified form of the Miami model⁷ to calculate NPP, which they reduced to fit a theoretically derived NPP distribution for regions outside the tropics, whereas King *et al.*⁸ found that no such modification was required for their study region, 64–90° N, when compared with available observations of NPP. No attempt was made to track carbon through the storage pools, a critical factor in estimating carbon sequestration^{8,9}, so the mass of carbon available for oxidation was not taken into account. Much of the carbon uptake identified by Dai and Fung is probably already back in the atmosphere, making their estimate an upper bound for the magnitude of this biospheric sink.

Dai and Fung's result is much more sensitive to climate change than that found by Melillo *et al.*¹⁰, who use a more detailed model to predict NPP. Melillo *et al.* found very little change in NPP associated with five different climate forecasts derived from doubled-CO₂ climate model runs. These all include substantial increases in temperature and precipitation. Several studies^{9–11} concluded that changes in the atmospheric CO₂ concentration, rather than changes in climate, produce the greatest change in NPP, particularly in the tropics, and the largest biospheric sink for carbon. Investigations of the long-term change in carbon uptake by the biosphere must also take into account how vegetation patterns will change in response to climate^{10,12} and decisions on land use.

Until we can do such an analysis, predictions of future CO₂ concentrations and the efficiency of policies directed at stabilizing atmospheric CO₂ must remain questionable. □

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Spiritual journey

LAST week Daedalus proposed a way of measuring the weight of the soul. It required the subject to die in an instrumented container whose mechanical constants were continually computer-refined from transducer data. As the soul left the body, its recoil would reveal its direction, velocity and spin; the change in weight would give its mass. Traditional theology is silent on the spin of the soul, though it may predict that the soul of a sinner would depart downwards, and might weigh less than that of a righteous believer. Such experiments could settle this and other speculations. Does everybody have a soul? Do its mass, spin and speed mirror its owner in any way? Does it leave at the moment of clinical death, or can it depart earlier, leaving the body in a soulless state of animal existence? Indeed, do animals have souls, and if so which ones, and how do the mechanical properties of their souls vary with species? Many have died, or inflicted death, for much less fundamental points of theology.

Even so, an extensive programme of inherently lethal experiments is ethically worrying. The converse approach may be more acceptable: that of detecting the weight-gain and mechanical impact of a soul entering a female subject as she becomes pregnant. The study need not invade anyone's sexual privacy. Many theologians argue that the soul enters the fetus a week or so after conception. This is the point when it has acquired individuality; for if divided, it will no longer develop into twins. It is clearly worthwhile to establish this moment accurately. If the soul turns out to enter the fetus quite late in pregnancy, the religious arguments against contraception and early abortion will be neatly disproved.

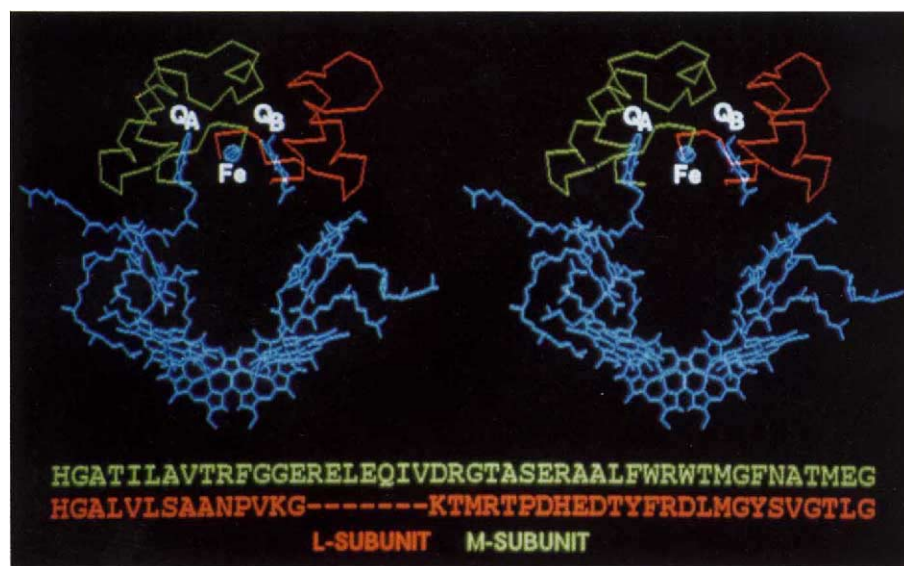
Daedalus, however, would like to detect the soul *in situ*, not merely at its moment of arrival or departure. He points out that normal material objects have identical inertial and gravitational masses. The soul may not. In that case, a creature with a soul would fall *in vacuo* with an acceleration slightly different from *g*, and as a compound pendulum would swing at a slightly different frequency from the equivalent distribution of dead matter. The effect would be extremely small. It might be detectable as a slight orbital deviation in a manned spacecraft, due to the anomalous inertial mass of the souls aboard it. But near-Earth orbits, perturbed by atmospheric drag, are probably too uncertain to reveal the effect. Accordingly, it provides perhaps the only scientific justification for a manned spaceflight to Mars. David Jones

Atavistic reaction centre

SIR — Evolutionary models of the photosynthetic reaction centre in the purple bacteria propose that an ancestral protein (a putative homodimer, X_2) may have had two parallel electron transfer pathways, each of them capable of reducing a quinone molecule¹. Over time, the two identical sequences are thought to have diverged and created a heterodimeric structure, wherein the two quinone binding sites became more specialized and the two quinones (Q_A and Q_B) accepted electrons in series^{2,3}. The recent discovery of apparently homodimeric reaction-centre complexes in the non-purple bacteria *Heliobacillus mobilis*⁴ and *Chlorobium limicola*⁵, combined with the fact that

whether gene duplication is a credible hypothesis for the origin of the present-day reaction centre in the purple bacteria.

To answer these questions, we have made a reaction centre in the bacterium *Rhodobacter capsulatus*⁶ in which the sequence comprising the primary quinone (Q_A) binding site is duplicated, so that the native Q_B sequence is deleted (see figure). In the original mutant (' $Q_A Q_A$ '), a continuous segment of 42 residues from the M subunit (Thr^{M220} to Glu^{M261}) replaces a segment of 35 residues in the L subunit (Leu^{L193} to Leu^{L227}). Because the $Q_A Q_A$ mutant is photosynthetically inactive, we used photosynthetic growth to select for revertant phenotypes. Analysis of the



Stereo pair of a portion of the X-ray crystallographic structure of the *R. viridis* photosynthetic reaction centre⁸. This pigment-protein complex has two quinone binding sites that are structurally related by a pseudo- C_2 symmetry axis; however, sequence differences in each binding pocket result in significant differences in their chemistry. The secondary quinone binding site (Q_B) is formed mainly by the L subunit (red), which differs in structure from Q_A . The Q_A pocket is mostly associated with the M subunit (green). The α -carbon backbones of the L- and M-subunits in the quinone binding sites are shown, beginning with two of the residues (His^{L190} and His^{M217}) which ligate the ferrous iron (Fe). These two protein segments end on opposite faces of Q_B and Q_A , respectively, at residues Gly^{L228} and Gly^{M262}. The bacteriochlorophylls and bacteriopheophytins on the active (A) branch are on the left. Those of the inactive (B) branch are on the right. Despite poor overall sequence identity, the ends of the two segments are located in nearly equivalent positions with respect to rotation about the C_2 axis. Engineering of restriction sites at both ends facilitates replacing the His^{L190}–Gly^{L228} Q_B segment with the His^{M217}–Gly^{M262} Q_A segment, generating the $Q_A Q_A$ mutant. The bidentate Glu^{M232} ligand to the Fe is located in the region that aligns with a gap in the L-subunit sequence. The two compensatory mutations found in the photosynthetically competent revertant of $Q_A Q_A$ (mutant A6D1) are interposed between Q_B and H_B (Met^{M144}→Ile, Ala^{M145}→Ser; graphics not shown).

the two quinones in some of the purple bacteria are chemically identical but nevertheless functionally different, raises the possibility that evolutionary selection for more efficient electron transfer led to a sequence divergence within the purple bacterial reaction centre; eventually forming the heterodimeric L- and M-subunit core complex. We therefore wished to determine how much of this protein asymmetry is essential for photosynthesis, and

complete L- and M-subunit sequences from the $Q_A Q_A$ mutant and one of the photosynthetically revertants confirmed that the L-subunit gene in the $Q_A Q_A$ construction contains a duplicated 135-base pair *NsiI*–*Bam*HI insert from the M subunit (which encodes most of the Q_A site). One of the revertants (denoted A6D1) is photosynthetically active even though it also has no Q_B -type sequence and contains 44 mutations relative to wild type.

The A6D1 revertant retains the Q_A insert intact, but in addition has two compensatory mutations in the M subunit: Met^{M144}→Ile and Ala^{M145}→Ser. In the structure of the *Rhodospseudomonas viridis* reaction centre as determined by X-ray crystallography⁷, these two residues are closest to the ring V (C_9) keto-group of the bacteriopheophytin (Bphe) on the inactive pathway (H_B) (ref. 8), and are somewhat further away from the wild-type Q_B site. The reason these two mutations are compensatory is not evident from the structure of *R. viridis*, but if H_B were inactivated during the process of divergence of the two pathways, it is interesting that in the symmetrized mutant the compensatory mutations are near this Bphe. We could speculate that a serine near H_B introduces a new hydrogen bond to the Bphe, and that somehow the inactive electron acceptor has been re-activated.

The fact that the duplicated Q_A insert remains intact in the revertant indicates that moving this M-subunit motif into the L subunit does not interfere with assembly of the reaction centre, even though the Thr^{M220}–Glu^{M261} sequence contains an extra potential ligand to the non-haem Fe at Glu^{M232} (ref. 8). Moreover, residues known to be involved in Q_B protonation^{9,10} are absent. The A6D1 reaction centres have a room-temperature optical absorption spectrum and a light-minus-dark absorbance difference spectrum that are indistinguishable from the wild type. Following an actinic flash, however, the kinetics of the charge recombination reaction between the reduced quinone(s) and the oxidized primary donor show no evidence of a Q_B -type acceptor, even in the presence of added quinone. SDS-PAGE of the isolated A6D1 reaction centre shows that the third subunit (H) is present.

Because various previously engineered point mutations in the Q_B pocket^{6,9–11} inactivate reaction-centre photochemistry, it has been reasonable to assume that a Q_B -type pocket is essential. But the viability of the A6D1 mutant demon-

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strates that an alternative structure, in which sequences for the two quinone pockets are undifferentiated, is nevertheless functional enough to support photosynthetic growth. This simplified reaction-centre structure provides an opportunity to study the directionality of electron transfer in a system in which numerous contributions to the L/M structural asymmetry have been eliminated. It offers as well a further indication that the present-day purple bacterial reaction centre could have evolved from a more symmetrical homodimeric ancestor.

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Naming names

SIR — Patterson¹ takes exception to our usage of the terms 'bird', 'dinosaur' and 'avian' in reference to a new Mongolian fossil archosaur, *Mononykus olecranus*². Some of Patterson's criticisms stem from misrepresentation of our paper, while others reflect fundamental disagreements. Patterson suggests that we consider *Mononykus* a member "of birds (a monophyletic group) and dinosaurs (a nonmonophyletic group)". We did not say this; throughout the paper we consider dinosaurs to be monophyletic because birds are members of this group. Birds are a kind of dinosaur, and Patterson's neologism "dinosaur-like birds" is therefore nonsensical.

A second issue raised by Patterson concerns the criteria for clade demarcation in groups comprising both extant and fossil taxa. Hennig³ proposed three methods for recognizing clade boundaries, the first two based on relationships and the third based on 'essential' characters. We agree with Hennig and Patterson that the third criterion leads to confusion in formal definitions of taxa. We used the term 'bird' to refer to members of the Avialae, and Patterson suggests that in so doing we used this third criterion, thus leading to confusion. Because we defined our taxonomic groups phylogenetically and pointed out that our usage of the term 'birds' was in the casual, rather than the formal taxonomic, sense, we do not see where confusion could arise.

We followed the work of Gauthier and others⁴⁻⁶ in considering the formal taxonomic term Aves to comprise only those taxa inferred to have descended from the most recent common ancestor of extant birds (method 1 of Hennig). Patterson instead advocates the inclusion in extant groups of all extinct taxa more closely

related to the group than to its sister-group (method 2 of Hennig). We prefer the so-called crown-group approach because, of the two methods, it most closely reflects longstanding usage of the content of Aves. The method advocated by Patterson would require that Aves include not only birds but pterosaurs and ornithischian and sauropod dinosaurs^{4,7}. Most ornithologists would have difficulty expanding their expertise to these groups.

In advocating Hennig's method 2, Patterson suggests that method 1 may provide misleading dates for the first appearance of the group. Although a molecular biologist would be misled if she or he thought that the age of the oldest member of the crown group chronicled the split between it and its extant sister-group, this would have more to do with sloppy scholarship than with any pitfalls in method 1. Method 2 would be similarly misleading if the molecular biologist asked a different question (for example, concerning the radiation of the living taxa rather than when they split from their sister-group). Because biologists are free to ask many different questions, we do not see that method 2 offers any advantages to guiding unsuspecting molecular biologists along the path towards enlightenment.

Finally, Patterson is concerned at the proliferation of formal taxonomic names for groups based on 'stem' species and their crown groups, such as the group Metornithes we named in our paper. We do not agree that this creates a problem, especially in an age when large databases can be manipulated with ease. Once a group is recognized to be a clade by the identification of derived characters diagnosing it, we see no justification for ignoring it merely because it includes extinct taxa. In our view, the use of a single name like Metornithes is preferable to the cumbersome "group composed of taxa x, y, and z". As we expand our knowledge of the biological world, names are needed to express our understanding of its diversity⁶. We believe that having names for all clades is more important than the practicalities Patterson prefers.

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PATTERSON REPLIES — Norell *et al.* and I differ in our approach to this problem, as shown by their second paragraph above.

They object to my calling dinosaurs a nonmonophyletic group, and write "we consider dinosaurs to be monophyletic because . . . birds are a kind of dinosaur".

It is generally agreed that the main impact of cladistics is in revealing and eliminating paraphyletic assemblages¹, "groups" which turn out to lack characters and reality. When dropped from scientific nomenclature, the names of paraphyletic groups are often retained merely as colloquial terms (for example, fishes, invertebrates). But there is always another possibility: a named paraphyletic assemblage can be salvaged, or made monophyletic, by expanding it to include the monophyletic group(s) whose exclusion caused the paraphyly. Reptilia (paraphyletic) might be made monophyletic by expanding it to include birds and mammals, for example, or Invertebrata might be made monophyletic by including vertebrates. One might then say "mammals (or birds) are a kind of reptile" or "vertebrates are a kind of invertebrate". Whether anything is communicated by such statements is not for me to say. But that is the procedure that Norell *et al.* recommend, with the difference that the paraphyletic group (dinosaurs) is extinct.

Norell *et al.* criticize the method I recommended² (the total-group) because it would force or encourage ornithologists into "expanding their expertise" to cover pterosaurs and dinosaurs, which would be included within Aves by that method. But mammalogists do not feel obliged to be expert on multituberculates, nor ichthyologists to be expert on pholidophorids, though they agree that those extinct animals are respectively mammals and teleosts. If ornithologists have to acknowledge that, when the fossil record is brought into the system, Aves includes dinosaurs and pterosaurs, I believe that the modification is less damaging than the course recommended by Norell *et al.*, which is that ornithologists should acknowledge that their stock-in-trade is, after all, "a kind of dinosaur".

In the 150 years since Richard Owen named Dinosauria for a few bones of *Iguanodon*, *Megalosaurus* and *Hylaeosaurus*, the result of the efforts of palaeontologists has been to discover what dinosaurs are, not what birds are. Perhaps surprisingly, dinosaurs turn out to be kinds of bird, a theory of relationships that is most clearly and economically expressed by including them within Aves.

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Smart collagen in sea lilies

SIR — Sea lilies (Echinodermata; Ctenoidea) are sedentary animals with a large crown of suspension-feeding arms and a jointed attachment stalk. The stalk can maintain an erect attitude or bend to permit changes in the orientation of the crown^{1,2}. Despite this adaptability, the stalk lacks any form of muscle³. It has therefore been suspected^{4,5} that the ligaments holding together its skeletal elements are 'smart' connective tissues. These tissues, also called mutable collagenous tissues, are unique to echinoderms. They can undergo drastic, nervously

between most of the ossicles, the only exception being the junction between each nodal and the internodal below it which is a rigid joint specialized for autotomy. All these joints are connected by collagenous ligaments³.

We performed tests on short lengths of stalk consisting of 7–9 internodals and including only mobile articulations. They were attached to a transducer as shown in Fig. 2a and subjected to a load of up to 100 g which tended to bend the stalk upwards. When a constant load was first applied, the preparations showed an immediate deflection which stabilized within a few seconds. When flooded subsequently with medium containing an elevated potassium ion concentration which would be expected to depolarize neural elements, such preparations exhibited a further deflection and then stabilized again (Fig. 2b).

Elevated K^+ concentrations had no effect on preparations which had been immersed in a sea water solution of the anaesthetic propylene phenoxetol, although they became responsive again after a wash in normal sea water. The preparations were also subjected to a stepwise increase in load before and after exposure to elevated K^+ concentrations. Excess K^+ increased the flexibility of the preparations, that is, the amount of deflection under a given load, an effect that could be reversed by returning them to normal sea water (Fig. 2c). Such potassium-induced alterations in mechanical behaviour can be due only to changes in the stiffness of the ligaments between the

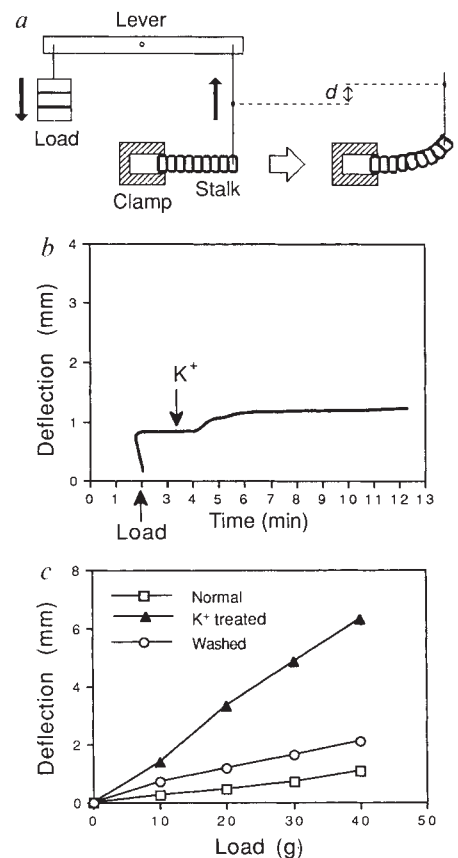


FIG. 2 a, Lengths of stalk were clamped rigidly at one end and the other end was attached to the lever of an isotonic transducer. Loads applied through the lever caused an immediate upwards deflection (d) of the stalk. b, Tracing of a curvilinear oscillograph recording of deflection against time for a typical preparation under constant load. The preparation was subjected to a load of 5 g which caused an immediate deflection, then flooded with 0.56 M potassium chloride (K^+) which caused a further deflection. c, Excess K^+ caused a reversible increase in the flexibility of internodal preparations. Typical results from a single preparation are given. Before treatment this was subjected to a load which increased by 10 g increments every 30 s. This procedure was repeated after the preparation had been immersed in 0.56 M KCl and then again after a prolonged sea water wash.

mediated changes in stiffness, switching between solid and semi-fluid states in less than a second^{6,7}. We report here results of experiments which confirm that 'smart' connective tissues are indeed present in the sea-lily stalk.

Specimens of the sea lily *Cenocrinus asterius* (L.) were collected at a depth of 600 m by the *Johnson-Sea-Link I* submersible at Egg Island and Chub Cay, Commonwealth of the Bahamas. The animals were kept in a darkened aquarium at 10 °C on board *R/V Seward Johnson* and used within 4 days of capture.

The endoskeleton of the sea-lily stalk consists of a series of washer-shaped ossicles which in *C. asterius* are differentiated into nodals that carry jointed appendages known as cirri and internodals that lack cirri (Fig. 1). The flexibility of the stalk depends on the presence of mobile joints

stalk ossicles: it is highly unlikely that other soft tissues such as epidermis or nerves³ could contribute mechanically to the recorded responses. The reversible abolition of responsiveness to K^+ by anaesthesia suggests that these ions depolarize neural elements that influence the stiffness of the stalk ligaments. Similar neurally mediated responses have been demonstrated in mutable collagenous tissues in other echinoderms and are known to depend on changes in the cohesive properties of the molecular 'glue' that binds together the collagen fibrils in these tissues^{6,7}.

The sea-lily stalk acts primarily as a rigid strut keeping the main body of the animal well clear of the sea floor. However, the presence of mutable collagenous tissues enables its flexibility to be adjusted so that the body can be re-orientated in

response to changes in current strength and direction and thus maximise the food-collecting potential of its arms^{2,8,9}. The

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sea lilies are the only survivors of a wide diversity of stalked echinoderms which thrived in Palaeozoic seas¹⁰. Although all of these were heavily calcified, there is extraordinarily little evidence for the occurrence of muscles in most of them, even in their feeding appendages (equivalent to the arms of sea lilies)⁷. It is, however, highly unlikely that their lives were spent in a state of perpetual unresponsive rigidity or flaccidity. The stalk of modern sea lilies illustrates how mutable collagenous tissues, in combination with external agents such as gravity and water movements^{2,8,9}, could have conferred mechanical adaptability on these animals.

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Music and spatial task performance

SIR — In the report by Rauscher *et al.* of an improvement in standard age scores after listening to Mozart (*Nature* 365, 611; 1993), the histogram is drawn over only part of the range of experimental values, and lacks a graphical or text indication of the distribution of the observed results about the mean. However, it is possible to make a rough calculation of the maximum value of the standard deviation from the statistical analysis. Assuming that the distribution of the results is normal, the confidence limits within which the population mean is likely to lie can be found. The probability quoted by the authors that the 'music' and the 'relaxation' conditions are different is 0.002, and from this *t* can be estimated. Since *n* = 36, and the difference in means between the two conditions is 8, the standard deviation must be very small for the 'population' mean estimates not to overlap — certainly less than 8. But IQ-related properties generally are more variable than this.

Admittedly this 'back of envelope' calculation is full of assumptions. But it demonstrates the need for variation, as well as central tendency, to be indicated in comparisons of this type.

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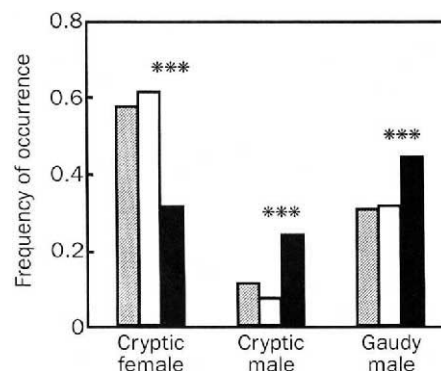
Risks of alternative mating tactics

SIR — Bright colours and conspicuous mating tactics may reduce survival by attracting predators^{1,2}. Evolutionary theory proposes that trade-offs between mate attraction and predator avoidance can promote the development of inconspicuous, safer, 'alternative' mating strategies^{3,4}. Our study of predation on different male colour morphs of the parrotfish, *Sparisoma radians*, challenges the generality of this supposition.

S. radians is a common inhabitant of Caribbean seagrass meadows. Like many other sex-changing fishes, females give rise to different male morphs that display a range of mating tactics⁵. Gaudy males of *S. radians* defend territories in which they actively court and mate singly with females, whereas cryptic males (that resemble females) form small, compact schools ($\bar{x}$ = 7 fish; *n* = 37 schools) that persistently follow and spawn with single females.

S. radians is the primary prey of the yellow jack, *Caranx bartholomaei*, representing 44 per cent numerically and 65 per cent by weight of 991 recognizable prey from 396 jack stomachs we collected in Panama between 1988 and 1992. Before the mid-afternoon spawning period of *S. radians*, females and both male morphs were eaten in relation to their availability in the population. In sharp contrast, both gaudy and cryptic males were found much more frequently than expected in the stomachs of jacks collected during the spawning period. Unexpectedly, cryptic males were 2.3 times more likely to be eaten than gaudy males when compared with their relative abundance. These differences were due neither to size-selective predation by jacks (data and analyses in the figure) nor to differential experience or predator avoidance by older gaudy males: the size range of gaudy males spans that of cryptic males, and large (> 11 cm) gaudy males are actually taken more frequently than small (6–11 cm) ones (χ^2 = 13.8; *P* < 0.001).

This shift over the course of the day from non-selective predation on sexually inactive *S. radians* to selective predation on spawning males, particularly cryptic males, provides a unique demonstration not only of sexual differences in the predation costs of mating but also of differences in such costs for different male mating tactics. Male mating behaviour, rather than colour pattern, must drive these predation patterns, since both types of males suffered higher predation only when sexually active. Conspicuous courtship and territorial behaviour probably draw attacks on gaudy males. We think that schooling invites predation



Frequency of occurrence of *S. radians* in the population (*n* = 1,269 fish from 18 trawls in 1992; open bars) and in the stomachs of jacks collected by handspear before (shaded bars) and during (filled bars) the spawning period of *S. radians* (*n* = 52 *S. radians* from 44 jacks and *n* = 262 *S. radians* from 180 jacks, respectively). The relative abundances of females and the two male morphs of *S. radians* in the population did not differ from those in 'pre-spawning' stomachs (χ^2 = 0.65; *P* = 0.72), but did differ from those in 'spawning-period' stomachs (asterisks, binomial comparison of proportions by fish type for population versus spawning period: *Z* ≥ 6.81; *P* < 0.0001). More cryptic and fewer gaudy males than expected were found within jacks collected during the spawning period (*n* = 194 males; χ^2 = 13.6; d.f. = 1; *P* < 0.001). Yellow jacks tend not to eat *S. radians* smaller than 6 cm, regardless of sex or colour, but consume *S. radians* larger than 6 cm in direct proportion to their availability (208 *S. radians* in six size classes; χ^2 = 4.22; d.f. = 5; *P* = 0.52).

upon cryptic males because schools are visually conspicuous and easy to exploit: jacks search for fish hiding in the seagrass and schools of cryptic males produce rich, male-biased 'patches' of prey. Preoccupation with obtaining females and repeated daily spawning by males may also boost their overall risk of being eaten.

To conclude, our data strongly support the general prediction that conspicuous male courtship imparts substantial risks⁶. They also reveal, however, that 'alternative' mating tactics by cryptically coloured individuals may actually be the riskiest of those available. This latter result is counterintuitive, given that both schooling and the expression of alternative mating tactics by cryptic morphs are generally thought to lower the relative risk of predation.

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Using artistic licence

Ian McClure

Art Restoration: The Culture, the Business and the Scandal. By James Beck with Michael Daley. John Murray: 1993. Pp. 210. £17.99.

THE conversation and restoration of great works of art invariably rouses controversy. Fiercely critical attacks on the cleaning of paintings in the National Gallery in London started in the 1850s when a committee of inquiry in London was set up. In the 1960s the controversy was continued in the *Burlington Magazine*. Then, in her book *The Ravished Image* (Weidenfeld & Nicolson: 1985), Sarah Walden attacked English and American approaches to the cleaning of paintings, suggesting they relied more on 'scientific' objectivity than aesthetic judgement. Now, in *Art Restoration*, James Beck and Michael Daley repeat the attack on the conservation profession, holding it to be at the mercy of scientists and attribution-seeking curators.

The book was written as a result of Beck's concern over the cleaning of Michelangelo's ceiling of the Sistine Chapel and the restoration, completed in 1988, of the Brancacci Chapel, particularly the scenes painted by Masaccio (see illustration). Before publication, Beck's criticism, widely published in the Italian press, of the cleaning of the marble tomb and effigy of Ilaria della Carretto, resulted in his being sued unsuccessfully by the conservator. This forms the prologue to the book. Meanwhile his coauthor, Daley, moves the scenario back to familiar territory, the National Gallery in London, making quite specific attacks on the cleaning methods used and the research undertaken by its conservation department.

The authors promise to expose "the Culture, the Business and the Scandal" of the international community of conservators, curators and large companies anxious to sponsor glamorous and dramatic conservation projects. Finally, Beck proposes a huge bureaucratic edifice to monitor all future conservation projects and promotes Artwatch International, of which he is founder and director (Daley being the British director), as an international watchdog.

Although many conservators share Beck's alarm over some issues, such as evidence that funding seeks high-profile conservation projects, the authors' desire to be included in the conservation debate is seriously undermined by selective and misleading use of evidence. There is no carefully argued plea for minimalist intervention, but rather, polemical journalism that uses all the tricks of the trade to make a point. For example, Beck shows

photographs of the head of Ilaria before and after cleaning to demonstrate the damage to the modelling as a result of cleaning. The difference at first glance is alarming as the head appears to have lost detail. Closer inspection, however, shows that the head in the first photograph, before cleaning, is lit steeply from the left, throwing the modelling into sharp relief. The head after cleaning is viewed with the lighting from above and to the right, illuminating the areas previously in shadow and obliterating the modelling of the face. This inconsistency in lighting renders the comparison meaningless. Similarly, later in the book Daley cites old photographs in black and white as evidence for later overcleaning, apparently unaware of the limitless possibilities for the manipulation of the photographic image in printing.

Central to the book is the suspicion of both authors of the role of science in cleaning paintings. Western conservators, they assert, are no longer trained artists with a developed, sensitive eye but scientists who seek confirmation from analysis and tests to undertake ruthless unsympathetic work, often applying untested materials to priceless works of art. They

point to unsuccessful projects in the past, thought then to be carefully conceived and carried out and ask how present-day conservators can assume their interventions will be longer-lasting. During the cleaning of Michelangelo's fresco on the Sistine ceiling, the conservators' openness in admitting that they had decided not to continue their initial use of an acrylic copolymer, Paraloid B72, to protect and saturate surfaces, is cited as confusion rather than as an example of laudable caution in continually reassessing the materials and techniques used.

They quote anecdotal theories explaining Michelangelo's technique but dismiss the analytical work undertaken by the conservation team. Beck and Daley believe that layers of glue applied to the ceiling were applied by Michelangelo to tone down and enhance the modelling, rather than being later attempts to resaturate the colours rapidly becoming covered with soot and dirt. The authors argue that because the application of glue is not mentioned in the records of subsequent work on the ceiling, it must have been applied by the artist. Early conservation records are notoriously inaccurate and glue layers are found as coatings in early restorations of paintings. Part of the reason for conserving the ceiling was the detection of flaking paint. That brittle and hygroscopic glue layers will inevitably and increasingly cause flaking of the paint layer is well known to conservators but dismissed here. The non-specialist is left confused and misinformed.

It is perhaps the chapter on the British



The Expulsion of Adam and Eve, by Masaccio, before (left) and after restoration.

National Gallery that most clearly reveals the nature of this book. The gallery was probably the first national collection to make public its conservation work, both by exhibition and by publications, principally the *Technical Bulletin* published annually since the 1970s. Daley makes use of information from this source yet derides those who wrote it. In his condemnation of the work done on Canaletto's *Stonemason's Yard* he seeks to ridicule the scientific approach by juxtaposing the expressive quotations by Matisse and Van Gogh on their use of colour with the conservator's precise description of Canaletto's varying mixtures of pigments to produce different greens. This demonstrates, although Daley seems unaware of the fact, Canaletto's skill in painting different effects at a time when the range of greens available to artists was very limited. The precise analysis of Canaletto's palette is of very great value to those who are studying and researching his paintings.

When Daley cites the research of P. L. Jones that suggests that solvents inevitably leach paint films, he fails to mention that the samples used were freshly painted and immersed totally in solvent for considerable periods of time. This is totally unlike the usual practice of varnish removal which entails passing a small solvent-moistened swab over the paint surface. Other assertions in the chapter, particularly the removal of a layer of glaze from Bramantino's *Adoration* display a lack of understanding of the techniques used to detect overpaint. Painting a shadow, as Daley suggests, because the connoisseur thinks it should be there is ethically untenable. But if you accept this type of intervention, all works become subject to the whims of contemporary taste in a way more distorting than trying to present paintings unencumbered by later repainting and the effects of discoloured varnishes.

All conservators should welcome debate and reassessments of their methods. But the debate should be informed and balanced, especially as the public shows an increasing interest in the past and its preservation in the face of increased pollution and other destructive agents. The reason for the public's interest can hardly be explained by the increased size of television sets or by the increased size of "the average family car in Europe" as the authors remarkably suggest. The spirit of masterpieces, the authors fear, is removed by conservation and scientific analysis. Readers need only visit the exhibition of the recently restored Wilton Diptych, currently at the National Gallery in London, to see how the reverse is true. □

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History is also about maps

D. H. Maling

Flattening the Earth. By John P. Snyder. University of Chicago Press: 1993. Pp. 365. \$45.00, £35.95.

THERE is an unusual background to the writing of this book. In 1978, the late Brian Harley and David Woodward first discussed preparation of a definitive 'history of cartography'. This massive work was expected to occupy six volumes, requiring many years for completion. The first volume, dealing with cartography in prehistoric, ancient and medieval Europe

greater work, Snyder's book stands out as a very important contribution to the subject. This is to be expected, for his earlier work demonstrates his encyclopaedic knowledge of the subject and its literature as well as his considerable mathematical ability. *Flattening the Earth* is, moreover, one of the few serious attempts to consider the overall history of the subjects as this is known to European and North American scholars. Other studies have been too restricted either in time or in subject.

The arrangement of the text is chronological, covering the emergence of map projections: classical to renaissance; in an age of mathematical enlightenment (1670–1799); in the nineteenth century; and in the twentieth century. Each section is further subdivided, first to demonstrate the continuity of the subject and later use of projections described or used in an earlier era; and second to summarize new discoveries and their applications.

John Snyder used to be a chemical engineer with an interest in maps. In those days he published some work on map projections and the history of cartography, but it was not until he had retired from work in the pharmaceutical industry that his important mathematical contributions started to appear. The first was the successful attempt to devise a conformal projection for use with complete strips of the Landsat Multispectral Scanner and the Thematic Mapper. He tackled the rather difficult problem of devising a suitable system to combine a skew oblique presentation of the spheroid (the Earth) with the variations in scanning the width of each swath made by the Landsat satellite. The result was the Space Oblique Mercator (or SOM) projection, most of the

details of which were published in a couple of US Geological Survey publications in 1978 and 1981.

As a result of his work on SOM, Snyder soon found himself working full-time for the US Geological Survey. He stayed there for a further decade and finally retired quite recently. It has been a remarkably fruitful period in the renaissance of mathematical cartography in the United States where, with the important exception of Waldo Tobler's work at Michigan, it has passed through a long period of inactivity. At the latest count, Snyder has published 30 papers and more extensive manuals, with half a dozen others in which he has been a joint author. Moreover, this prolific output has rubbed off on the younger generation and there are now an increasing number of contributions to the *American Cartographer* (now *Cartography and Geographic Information Systems*) which have clearly been inspired

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Mappa Mundi — thirteenth-century world map at Hereford Cathedral, England.

and the Mediterranean, was published in 1987. The next, comprising part of Volume 2, and concerned with Islam and southern Asia, appeared in 1992. The second part of Volume 2 is due to be published soon. It was intended to introduce the history of map projections, in appropriate places in the text, and in 1982 the editors invited John Snyder to contribute all these sections.

It seems, however, that they did not anticipate such a prompt response. Snyder's manuscript was completed long before they were prepared to use and edit the material. In his preface he comments: "Sensing my restless nature, Dr Woodward kindly suggested that I approach the University of Chicago Press about publishing the combined chapters as a single work, to be used later in the history volumes in a somewhat condensed form". This single work is the present book. Irrespective of its value as part of a much

by his lead and his enthusiasm for the subject.

I find little in this book to take issue with. The treatment follows the twentieth century continental European approach, and will therefore be accessible to cartographers on both sides of the Atlantic. The numerous illustrations are clear and consistently presented. Indeed, this is the most comprehensive collection of diagrams of the projections used in world mapping and is even more complete than the splendid *Album of Map Projections* (by Snyder and P. M. Voxland) published by the US Geological Survey in 1989.

One limitation is that the applications

are largely confined to European and North American literature. Apart from the Arab contribution, other cultures seem to have been ignored. For example, there is no mention of the early Chinese work unearthed by Joseph Needham, and published in Volume 3 of his monumental *Science and Civilisation in China* (1959), where he described the use in AD 940 of the Tunhuang star chart which represents the independent discovery of Mercator's projection by Ch'ien Lo Chih more than 500 years earlier. □

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Dawning of agriculture

Jared M. Diamond

Domestication of Plants in the Old World.

Second Edition. By Daniel Zohary and Maria Hopf. *Oxford University Press (Clarendon Press): 1993. Pp. 278. £35, \$52.50.*

THIS book is of much wider significance than its title suggests, as plant and animal domestication is important to two diverse groups of scientists. For evolutionary biologists, changes under artificial selection yield clear insights into evolutionary processes. Hence Darwin devoted the first chapter of his *Origin of Species* to variation under domestication. For students of human society, domestication launched the chain of developments leading to complex societies and thus to writing, metallurgy and empires. Geographical differences in domestication probably contributed to the widely differing trajectories and rates of development of societies in different parts of the world.

Among the world's centres of domestication, the one in which plant domestication is best understood is Southwest Asia (alias the Near East). That is fortunate, because Southwest Asia may be the earliest centre of domestication, as well as of writing and other marks of civilization. For most crops of Southwest Asia, the ancestral wild species has been identified by comparative morphology and genetics, and the ancestor's wild distribution and ecology are known. For many of these crops, the time and place of domestication can be pinpointed by plant remains from hundreds of carbon-dated archaeological sites spanning the past 12,000 years. The outcomes of crosses of the crop with its wild progenitor and with related wild species have been studied, and chromosomal, genetic, morphological and physiological changes that occurred under domestication have been identified.

The summarizing of this information in

the first edition of Zohary's and Hopf's book in 1988 made it the outstanding account of plant domestication for any area of the world. The second edition incorporates the accelerating advances of the past five years. In the accounts of vegetables, fruit and nut trees and dye crops, the added information makes this revision virtually a new book. Much new detail has also been added to the treatments of cereals, legumes, oil crops, fibres, condiments and wild-collected fruits.

The book is organized into two main sections. First, for each of 78 crops or plants, grouped in the above-mentioned categories, the wild relatives, archaeological finds and evolutionary changes are summarized. The second section summarizes plant remains from 150 selected archaeological sites spanning the Old World from India west to Britain, and from Egypt north to Finland. A brief final chapter provides some general conclusions.

This book is indeed a 'mine of information'. An enormous and diverse body of important results is digested and presented economically, in a form that should encourage other authors to mine it and apply the results to their own fields. Just three examples must suffice.

First, the map on pages 232–233 uses a compact set of symbols to depict dates for the centrifugal spread of six early crops from Southwest Asia west across Europe and east to India. Details of this map will surely also be scrutinized by linguists concerned with the spread of Indo-European languages, and by population biologists concerned with the genetic origins of modern Europeans.

Second, the same 'package' of eight or nine crops that founded agriculture in Southwest Asia founded it later in Europe, Egypt and the Indus Valley. Evidently, these so-called founder crops and the founder domesticated animals

associated with them constituted a powerful economic package that diffused rapidly along Eurasia's East–West axis. The founders' spread pre-empted independent domestications of the same crops outside Southwest Asia, as well as pre-empting domestication of other potentially domesticatable related species within Southwest Asia. Identification of chromosomal types and of mutant changes under domestication shows that, for most of the founder crops, all extant varieties are descended from a single domestication event. In contrast, spread of crops along the New World's North–South axis was much slower, with the result that the same species or related species were repeatedly domesticated independently in South America, Meso-America and eastern North America.

Third, the main sequence of plant domestication in Southwest Asia began with predominantly self-pollinated but occasionally cross-pollinated annuals (the founder cereals and legumes), proceeded through vegetatively propagated fruit trees, and led to fruit trees requiring mastery of difficult grafting techniques. In parallel with, or late in, this sequence came the evolution of weeds infesting fields of these primary crops; the weeds became crops in their own right, as in the case of oats and, probably, lettuce. These sequences fit well with evolutionary perspectives that view plant domestication as an initially unintended outcome of human harvesting of wild plants. The relationship between unconscious selection by humans and the resulting diverse changes under domestication is often transparent. For example, wild-pea pods, which disperse their peas by popping open, evolved by a single recessive mutation into domesticated peas with pods that do not pop open, because peas from popped pods would be lost and could not be gathered by human harvesters.

For anyone concerned with Old World plant domestication, this book in its revised edition will become the main reference. It will also be essential reading for many scientists who have no direct interest in the Old World or in plant domestication, but whose subject matter grows out of those subjects. That includes especially historians, cultural anthropologists, archaeologists, linguists, evolutionary biologists and students of human genetics and diseases. For botanists and agronomists concerned with plant domestication elsewhere in the world, Zohary and Hopf's synthesis for western Eurasia will serve as the standard at which to aim, and as a basis for intercontinental comparisons. □

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The technological rainbow

Daniel S. Greenberg

Empowering Technology: Implementing a US Strategy. Edited by Lewis M. Branscomb. MIT Press: 1993. Pp. 315. £33.75, \$37.50 (hbk); £15.95, \$17.95 (pbk).

PRESIDENT Bill Clinton came into office pledging to use government to rejuvenate civilian industry in the United States, a method employed gingerly by his free-market, Republican predecessors. By the measure of money, bureaucratic turmoil and heroic goals, Clinton is indeed labouring furiously on his promise.

More than \$8 billion is to be shifted from the Pentagon to civilian research during the next few years, ending longstanding military dominance in federal research and development spending. The renowned financier of military innovation, the Defense Advanced Research Projects Agency (DARPA), has shed the 'D' in its title and is now charged with bankrolling research that spans military and civilian projects. Clinton has proclaimed plans to develop a clean, highly fuel-efficient automobile through a collaboration between America's 'big three' car manufacturers and government researchers. Federal laboratories that thrived on Cold War missions are being reorientated to industrial tasks. And with government money and orchestration, plus reassuring winks from the anti-trust authorities, industrial research consortia are being formed to work on technical problems common to particular sectors of industry.

Precursors of these activities can be found in the Reagan-Bush presidencies, regardless of their scorn for 'industrial policy' and derision of sheltered Washington bureaucrats trying to 'pick winners'. The difference is scale and enthusiasm — huge on the part of Clinton, in contrast to the halting steps and ideological misgivings of the previous administrations.

Cheers, with serious reservations, are expressed for the Clinton shift by the editor and principal author of this collection, Lewis M. Branscomb, whose range of senior-level service is rare even in professionally mobile America. Director of the Science, Technology, and Public Policy Program at the Kennedy School of Government, Harvard University, Branscomb was formerly chief scientist at IBM, director of the National Bureau of Standards and chairman of the National Science Board, the policy-making body of the National Science Foundation.

Clinton has correctly focused on the pace and scope of technology adoption as

a weak link in US industrial performance, Branscomb writes. But, he argues, although useful technologies are abundant, the policies and structures of the federal government are ill suited to the task of strengthening the link. The technical agencies with the big budgets are Cold War leftovers with ingrained wrong habits for dealing with civilian industry, he continues. But even as they and old-line civilian agencies try to change, he contends, the federal apparatus remains too modest and timid for the new era of global industrial competition. Branscomb calls for nothing less than a comprehensive mobilization of resources — schools, federal, state and local agencies, government laboratories, universities and so on — to instil technological vigour in US industry.

He puts particular emphasis on encouraging technological enlightenment in the hundreds of thousands of small companies that are generally below Washington's threshold of notice. A fellow author in the volume, Harvey Brooks, professor of technology and public policy (emeritus) at Harvard, suggests the creation of academic-industrial "buffer institutions" in which industry and universities could collaborate without "erosion of the academic culture". No sector or institution that might contribute to technological superiority is overlooked.

Despite his concentration on government, Branscomb's design for high-tech nirvana says the job of government is to facilitate, orchestrate and finance, not to lead.

Branscomb is both hopeful and pessimistic about government's capacity to handle this role. He points out, for example: "Most of the civilian agencies lack experience with investing in the industrial technology base and in industrial extension and information infrastructure; these programs can only grow with experience". And he warns that "[g]overnment officials must be industrially experienced and must recognize the idiosyncratic nature of specific technologies and of each particular high-tech industry". To do their part in the new industrial regime, "government officials will have to become much more sophisticated in technology, economics, and politics than was necessary to administer basic research support to universities and manage the government's own technological responsibilities".

Can the US government fill the role? The potential has been demonstrated, Branscomb argues, by DARPA's prescient underwriting of "pathbreaking technologies that created industries". The National Institutes of Health (NIH) also sowed the scientific seeds for the biotechnology revolution and its great industrial promise. The role of the government's technical managers in these achievements is not to be discounted. But DARPA and

the NIH were free to drench their fields of technical choice in money, with few questions asked: \$4 billion a year from the NIH for biotechnology and \$1.5 billion for DARPA's various projects.

One may be sceptical about whether government can muster the sophistication in technology, economics and politics prescribed by Branscomb. But closer technological relations between government and civilian industry are already here and are bound to increase, if only because of the lure of federal money. The effectiveness of these collaborations, however, is far from established, while their potential for becoming politically contaminated is always there. Furthermore, industry has good reason to be wary of government's steadfastness in science and technology, as the refugees from the political collapse of the Superconducting Super Collider can attest.

Branscomb urges patience and caution in developing the new era of technological collaboration. The participants must learn their way, he warns. But the dominant tone in *Empowering Technology* is that we must get on with it, quickly. However, the reality of the moment is that high-tech US industry seems to be picking up speed and profits on its own. The problem just dawning on America is that rising technological prowess kills jobs.

Robert M. White, president of the National Academy of Engineering, recently delivered a gloomy public address entitled "What Is at the End of the Technological Rainbow?" Large-scale job losses, he answered, and he went on to raise the possibility that contemporary technology is not conforming to its historic role of job creation — a startling observation from the chief of America's high temple of engineering. "Is our faith in historical precedent well founded?" White asked. "The answer", he said, "is that nobody knows."

Branscomb passes quickly over issues of the underside of technology, noting that "technology decreases the demand for labor in a static demand situation". His goal is industrial efficiency and productivity, and the promise is that they can add to general prosperity, as they have in the past. Perhaps they can. But wonder is mounting about what lies at the end of the technological rainbow. □

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New in paperback

■ *Cold Fusion: The Scientific Fiasco of the Century* by John R. Huizenga, Oxford University Press, £8.99. For a review see *Nature* 358, 291 (1992).

Human basophil degranulation is not triggered by very dilute antiserum against human IgE

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We have attempted to reproduce the findings of Benveniste and co-workers, who reported in 1988 that degranulation of human basophil leukocytes is triggered by very dilute (10^2 – 10^{120}) antiserum against IgE. The results were contrary to conventional scientific theory and were not satisfactorily explained. Following as closely as possible the methods of the original study, we can find no evidence for any periodic or polynomial change of degranulation as a function of anti-IgE dilution. Our results contain a source of variation for which we cannot account, but no aspect of the data is consistent with the previously published claims.

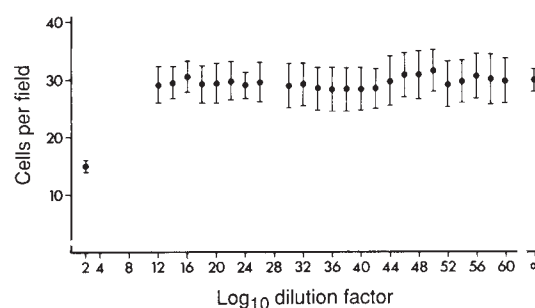
WE examined the effects of dilutions of anti-IgE from 10^2 to 10^{60} on human basophil degranulation. The original paper¹ stated that anti-IgE dilutions were effective only if vigorously shaken (succeeded) during preparation. We have compared the effects of succussed anti-IgE, unsuccussed anti-IgE and succussed buffer. The 10^2 dilution of anti-IgE produced consistent, significant degranulation, but dilutions from 10^{12} to 10^{60} , whether succussed or not, failed to cause degranulation. In Fig. 1, the data from all three treatment types (succussed anti-IgE, unsuccussed anti-IgE and succussed buffer) have been pooled.

FIG. 1 Mean cell densities (with standard errors) as a function of dilution for all the data. The data for succussed anti-IgE, unsuccussed anti-IgE and succussed buffer have been combined. For the buffer control (10^∞) and anti-IgE dilution 10^2 , $n=108$; for the other anti-IgE dilutions, $n=36$.

METHODS. Blood (120 ml) collected by venepuncture from a superficial ante-cubital vein was anticoagulated with heparin (1U ml^{-1}), EDTA- Na_2 and EDTA- Na_4 (each 2.5 mM) in saline (154 mM) containing 6% dextran T-70. Red cells were sedimented for 90 min at room temperature (21°C), and the leukocyte-rich plasma ($\sim 70\text{ ml}$) was recovered and centrifuged at $400g$ for 10 min. Pellets were resuspended in HEPES-buffered Tyrode solution (in g per l: NaCl, 8.0; KCl, 0.195; HEPES, 2.6; EDTA- Na_4 , 1.04; glucose, 1.0; human serum albumin, 1.0) containing heparin (5,000 units per litre) at pH 7.4 and recentrifuged. Pellets were resuspended in HEPES-buffered Tyrode solution and pooled before further centrifugation. The final pellet was resuspended in 1.5 ml HEPES-buffered Tyrode solution. Anti-IgE (goat anti-human IgE (Fc) from Nordic Immunology, UK; stored frozen at -75°C in $10\text{-}\mu\text{l}$ aliquots of 1 mg ml^{-1} in sterile deionized water) was serially diluted in triplicate by 100-fold in the range 10^2 to 10^{60} in HEPES-buffered Tyrode solution in polystyrene tubes. Between each dilution, solutions were mixed as required by the protocol by vigorous mechanical shaking (succussion) for 10 s. Mechanical shaking was standardized using a device (on loan from Nelson's Homeopathic Pharmaceutical Co.) with which the tube containing the solution would strike a rubber bung roughly 20 times per second. In some experiments, dilutions of HEPES-buffered Tyrode solution were shaken in the same way. The prepared solutions were taken to a separate room away from the laboratory and an independent person working alone applied a code to each tube using random number tables. The key to the code was sealed in an envelope and retained until the experiment was completed. The numbered tubes were then shuffled and returned to the person conducting the experiment. It was not possible for the person performing the experiment to know the contents of the tubes. To each coded tube, $5\text{ }\mu\text{l}$ CaCl_2 was added to give a final concentration of 5 mM, followed by $30\text{ }\mu\text{l}$ of the cell suspension

The only apparent and significant effect of treatment on cell density occurs at the 10^2 dilution of anti-IgE. Any effect of the higher dilutions would have to be very subtle.

Results from the three different treatment types are shown separately in Fig. 2. Separate controls were included in each experimental session and the means for each of the dilution ranges (ranges 1, 2 and 3; see Fig. 1 legend) examined are shown in Fig 2. Mean cell density appears to depend on dilution of anti-IgE, but the control cell density also varies between experiments, which demonstrates the need for appropriate controls. Without



prepared as described. The tubes were incubated at 37°C for 30 min, then centrifuged at $400g$ for 10 min at room temperature. Pellets were resuspended in $100\text{ }\mu\text{l}$ staining solution (100 mg toluidine blue and $280\text{ }\mu\text{l}$ glacial acetic acid in 100 ml 25% ethanol in deionized distilled water). Dense purple-staining (non-degranulated) basophils were counted using an improved Neubauer haemocytometer at $\times 500$ magnification. Cells were counted by a single, trained individual who recorded the number of cells in each high-power field and the number of fields, to provide a total cell count of ~ 100 per sample. To ensure that cells were counted within a reasonably short time after preparation, experiments were conducted in sessions, which consisted of 30 samples comprising triplicates of 10 different treatments. Each session was completed in a single day. The 10 treatments in a session were: the control of buffer alone (10^∞), the control low dilution of anti-IgE (10^2) and 8 different high dilutions. These high dilutions were either succussed anti-IgE, unsuccussed anti-IgE or succussed buffer. A total of 36 sessions were conducted as follows. Succussed anti-IgE (treatment A): 10^{12} – 10^{26} (range 1), repeated 5 times; 10^{30} – 10^{44} (range 2), repeated 5 times; and 10^{46} – 10^{60} (range 3), repeated 5 times; unsuccussed anti-IgE (treatment B): same three dilution ranges, repeated 4 times; succussed buffer (treatment C): same three dilution ranges, repeated 3 times.

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TABLE 1 Results of tests on mean differences and mean cell densities

Treatment type†	Dilution range	t-Tests* on mean differences		d.f. n_1	n_2	F-tests‡ on mean cell densities	
		Maximum t-statistic	Bonferroni bounds for P value			F_{n_1, n_2}	P value§
A	1	2.003	$0.116 < P < 0.926$	8	32	0.269	0.972
	2	0.888	$0.425 < P < 1$			0.317	0.954
	3	2.970	$0.041 < P < 0.329$			1.849	0.104
B	1	1.722	$0.184 < P < 1$	8	24	0.806	0.604
	2	1.089	$0.356 < P < 1$			0.792	0.615
	3	1.986	$0.141 < P < 1$			0.567	0.794
C	1	2.041	$0.138 < P < 1$	8	16	0.686	0.698
	2	3.050	$0.093 < P < 0.742$			0.683	0.701
	3	2.696	$0.114 < P < 0.915$			1.551	0.216

* The t-tests test separately for each dilution whether the mean difference is significantly different from zero. There are 8 such tests within each dilution range and the value given (mean control cell count – mean treatment cell count) is the maximum of these. The degrees of freedom are 4, 3 and 2, respectively, for treatment types A, B and C.

† The F-tests allow for an additive 'session' effect and test whether there are differences among the high-dilution and control means. The tests are based on 45, 36 and 27 observed means for treatment types A, B and C, respectively.

‡ Treatments: type A, succussed anti-IgE; type B, unsuccessful anti-IgE; type C, succussed buffer.

§ The F-tests are based on independent groups of sessions and their combined (Fisher) P values for treatment types A, B and C are all between 0.5 and 0.9.

these controls, the data could be interpreted in terms of an effect of high dilution on cell density. Such variation in controls has resulted in the customary practice of presenting the data as percentage degranulation rather than cell density. Davenas *et al.*¹ reported data in terms of percentage degranulation without showing the unmanipulated cell density data.

Figure 3 shows the same data as Fig. 2, but the ordinate is now percentage degranulation. As expected from the definition of percentage degranulation, values are distributed on either side of zero, in contrast to the data of Davenas *et al.*¹, in which there were no values of percentage degranulation below zero. The data in Fig. 3a do appear to reveal an effect of the higher dilutions of succussed anti-IgE. In these experiments there is a natural null hypothesis to test, namely the treatment applied to the cells produces a response which is not different from the response in the absence of a treatment. Because of this, statistical evaluation of the data relies on P values. Percentage degranulations do not have the expectation of zero even when the null hypothesis is true and so t-tests have been performed on differences defined as mean cell density of the control (buffer) minus the cell density of the treated sample.

The t-tests applied to the mean differences for the 10^2 dilution

of anti-IgE revealed that the differences from zero were all highly significant: $P < 0.0001$. The t-tests for the mean differences for the high dilutions are given in Table 1. None of the P values reaches the level of statistical significance, with the exception of the high dilution range for the succussed anti-IgE, for which the P value indicates borderline significance. Two-way ANOVA F-tests were performed on the high-dilution mean cell densities (Table 1). This test examines the hypothesis of no difference between dilutions, and in this case none of the P values reached the level of statistical significance. It therefore seems likely that the 'significant' t statistic in the highest dilution range for succussed anti-IgE is a chance result. This 'significant' effect was much smaller than anything reported by Davenas *et al.*¹. The nature of the degranulation assay is such that variability is large: it depends on counting a small number of cells (basophils constitute about 1% of circulating leukocytes) and requires a subjective assessment of stained cells. Also, for any given set of random numbers, it is expected that there will be occasions when the examination of randomness by statistical tests fails.

Davenas *et al.*¹ found effects that seemed to disappear and reappear at different dilutions. We therefore examined the data for polynomial trends or periodic cycles in mean percentage

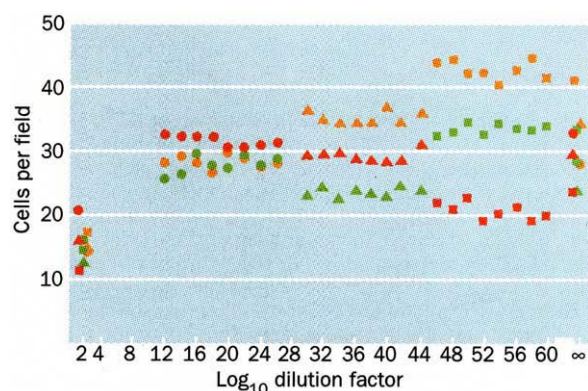


FIG. 2 Mean cell densities as a function of dilution for the separate treatments. Red, Succussed anti-IgE (treatment A), $n=15$; Green, unsuccessful anti-IgE (treatment B), $n=12$; Yellow, succussed buffer (treatment C), $n=9$. ●, range 1 data; ▲, range 2 data; ■, range 3 data. The standard error bars have been omitted for clarity, but for the high dilutions the standard errors were about ± 9 , which is fairly wide mainly because of between-session variation.

TABLE 2 ANOVA tests (P values) for differences between dilutions separately for each session

Treatment type*	High-dilution range		
	10^{12} – 10^{26}	10^{30} – 10^{44}	10^{46} – 10^{60}
A	0.34	0.028	0.38
(combined 'Fisher')	0.066	0.018	0.21
P value = 0.0027)	0.14‡	0.42	0.17
	0.0043§	0.42	0.21
	0.70†	0.80	0.40
B	0.56	0.25	0.27
(combined	0.27	0.97	0.27
P value = 0.086)	0.0073	0.76	0.16
	0.084	0.48	0.44
C	0.92	0.80	0.66
(combined	0.25	0.25	0.21
P value = 0.85)	0.65	0.91	0.71

ANOVA tests used the one-way F-test with (8, 18) degrees of freedom, using control and high-dilution treatments in each session; that is, the 10^2 dilutions were excluded.

* See legend to Table 1 for treatment types.

† The missing value in this session means that the F-test used (8, 17) degrees of freedom.

‡ See Fig. 4, Yellow. § See Fig. 4, Red. || See Fig. 4, Green.

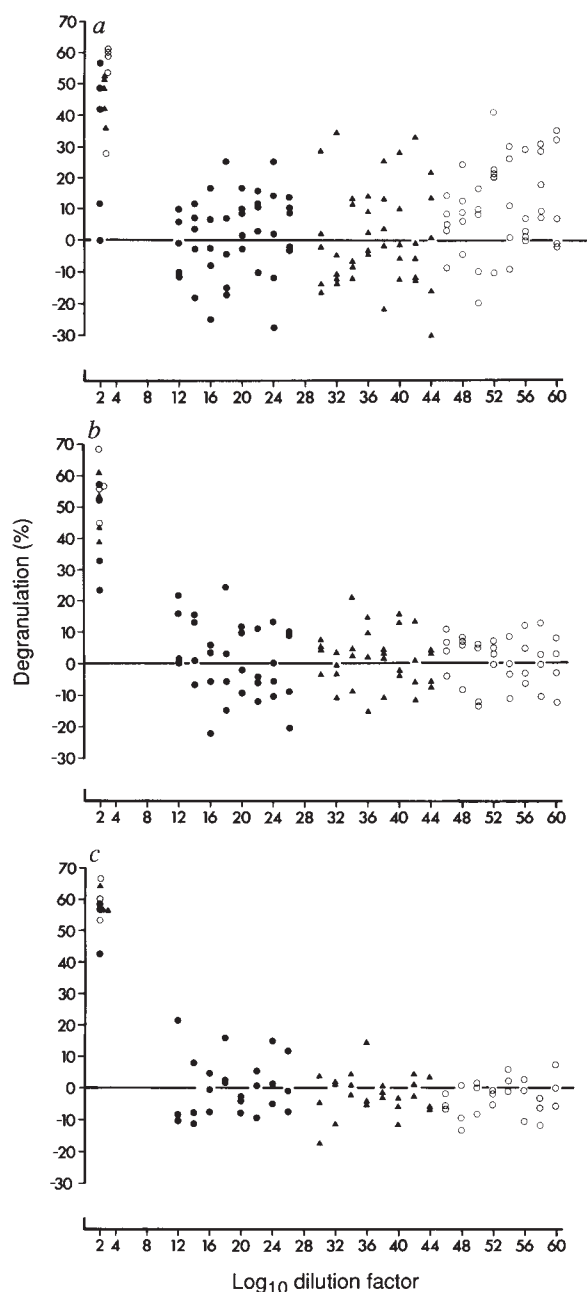


FIG. 3 Percentage degranulation of basophils as a function of dilution. Each point is the mean of triplicate determinations in a single experiment. a, Succussed anti-IgE (treatment A); b, unsuccussed anti-IgE (treatment B); c, succussed buffer (treatment C). ●, Range 1 data; ▲, range 2 data; ○, range 3 data. The percentage degranulation was calculated as $100 \times (1 - \text{treatment sample cell density} / \text{mean control sample cell density})$.

degranulations. With one exception, the P values for constant, linear, quadratic, cubic and periodic events with two, three or four cycles were greater than 0.1. The only convincingly significant feature ($P=0.0015$) was the linear component of trend for the highest dilution range of succussed buffer, which is inconsistent with Davenas *et al.*¹ and with conventional scientific theory.

Cell counts for fixed volumes of cell suspension should exhibit Poisson variation, possibly with some 'overdispersion' resulting from sources of variation such as counting or data recording errors, variations in volumes, and so on. In our data, each triplicate corresponding to a single dilution level within a particular experiment (session) might be expected to exhibit such extra-Poisson variation. This was quantified by using the χ^2 statistic to

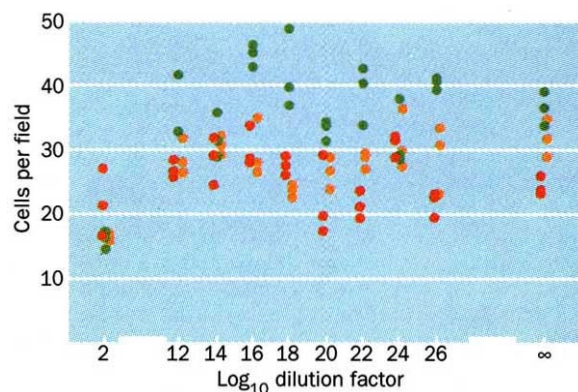


FIG. 4 Basophil densities as a function of dilution from single experiments showing the scatter of the triplicate values. Red, Succussed anti-IgE (treatment A); Green, unsuccussed anti-IgE (treatment B); Yellow, succussed anti-IgE (treatment C). See Table 2 and text for explanation.

compare the observed counts in each triplicate with the expected counts. The expected counts were calculated from the estimated mean count per field. Ten χ^2 statistics (with 2 degrees of freedom) were calculated for each 'session' (one for each triplicate) and summed to give a χ^2 statistic with 20 degrees of freedom. The 36 χ^2 values (one for each 'session') were generally in excess of 20, suggesting that the anticipated overdispersion was indeed present. Overall, the data suggest that the counting variance was about 1.5 times the pure Poisson variation.

According to conventional scientific theory, there should be no differences within a session between the control treatment and the eight high-dilution treatments. This can be tested separately for each session by applying the conventional one-way ANOVA F -test to the mean counts (cells per field) for each tube. The resulting P values in Table 2 are curious because the P values should, if the null hypothesis is correct, be uniformly distributed between 0 and 1. This is not the case for treatments A (succussed anti-IgE) and B (unsuccussed anti-IgE), for which the P values are collectively too small (Table 2). The triplicates appear to differ from one another. Figure 4 (red and green points) shows the data with the most significant P values of 0.0043 and 0.0073. These differences detected by the F -test are small, and the most obvious ones are shown in red and green. In contrast, the yellow points in Fig. 4 show the more typical case, in which the differences were not significant ($P=0.14$). There is no regular or reproducible pattern in this 'between triplicate' variation and it is a feature of both unsuccussed and succussed anti-IgE treatments. We do not think that this can be explained by the method of preparation of the dilutions and it is not attributable to repeated dilution effects. Although it is possible that these observed effects are a statistical artefact, some unidentified part of our experimental procedure might account for them. It is an interesting feature of our data but it does not, of course, lend any support to the findings of Davenas *et al.*¹, and serves once again to underscore the complexity of the analysis of variance in an assay of this type.

We have been unable to find any evidence that very high dilutions of anti-IgE, succussed or unsuccussed, cause any reproducible effects on the degranulation of human basophil leukocytes. □

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1. Davenas, E. *et al.* *Nature* **333**, 816–818 (1988).

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Efficient catalytic addition of aromatic carbon–hydrogen bonds to olefins

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The selective cleavage of carbon–hydrogen bonds in organic compounds is a critical step in many organic syntheses, and is particularly important in the conversion of hydrocarbons to useful organic compounds. An organometallic ruthenium complex can cleave C–H bonds in a variety of aromatic systems, leading to addition to alkenes by C–C bond formation. The catalyst operates with a degree of efficiency, selectivity and generality that will make it extremely valuable in organic synthesis.

METHODS for carbon–hydrogen bond cleavage by transition-metal complexes, which would provide a pathway to many useful organic compounds, have been sought for the past 30 years^{1–10}. The direct cleavage of a carbon–hydrogen bond, followed by new bond formation at that carbon centre, would constitute an ideal synthetic operation, without the need to sacrifice any extra functional groups, as is often the case. Although there are now many examples of stoichiometric carbon–hydrogen bond cleavage by various metals^{3–10}, the utility of these reactions in catalytic organic synthesis is limited by their low yields and/or low turnover number of the catalyst. Notable recent advances include zirconium-catalysed addition of α -picoline to propene¹¹ and ruthenium-catalysed coupling of pyridine, carbon monoxide and 1-hexene¹². Although the yields in these latter two cases were exceptionally high, the reactions also yielded by-products or a mixture of isomers, and are apparently applicable only to a few pyridine derivatives^{11,12}. Here we describe a ruthenium complex that catalyses the coupling of aromatic carbon–hydrogen bonds with olefins. This complex, $\text{RuH}_2(\text{CO})(\text{P}(\text{C}_6\text{H}_5)_3)_3$ (ref. 13), can bring about addition of the carbon–hydrogen bond at the *ortho* position of aromatic ketones to the double bond of olefins. The new catalytic reaction achieves the efficiency, selectivity and generality required for practical organic synthesis. The key to our success is the involvement of chelation assistance: the low-valent ruthenium becomes coordinated to the aromatic carbonyl group, positioning it for subsequent cleavage of the adjacent aromatic carbon–hydrogen bonds^{8–10,14,15}.

Experimental details

Reaction and catalyst. The new catalytic reaction is shown in Fig. 1. A typical experiment was carried out as follows. A mixture of 2 mmol of an aromatic ketone, 2–10 mmol of olefin and 0.04 mmol of $\text{RuH}_2(\text{CO})(\text{P}(\text{C}_6\text{H}_5)_3)_3$ in 3 ml of toluene was vig-

orously refluxed (oil bath temperature, 135 °C) for several hours to give the C–H/olefin coupling product, which was isolated (by bulb-to-bulb distillation) as an analytically pure sample. The ruthenium complex $\text{Ru}(\text{CO})_2(\text{P}(\text{C}_6\text{H}_5)_3)_3$ was equally effective, and $\text{RuH}_2(\text{P}(\text{C}_6\text{H}_5)_3)_4$ and $\text{Ru}(\text{CO})_3(\text{P}(\text{C}_6\text{H}_5)_3)_2$ were modestly effective as the catalyst. $\text{RuHCl}(\text{CO})(\text{P}(\text{C}_6\text{H}_5)_3)_3$ and $\text{Ru}_3(\text{CO})_{12}$ were not effective. Using $\text{RuH}_2(\text{CO})(\text{P}(\text{C}_6\text{H}_5)_3)_3$ as the catalyst, the reactions of various aromatic ketones with various olefins were examined. The results are shown in Table 1.

Efficiency. A remarkable feature of the present reaction is its efficiency, especially the high yields of the C–H/olefin coupling products. In many cases, almost quantitative yields of products based on both the starting materials are obtained (runs 8–13). This has never been attained before in the catalytic functionalization of unactivated C–H bonds. In previously known reactions, it was necessary to use one of the starting materials in excess (often as the solvent^{12,16}) in order to obtain higher yields. The molar ratio of reactant/catalyst is high enough for a synthetic reaction and the turnover frequencies of the catalyst found in runs 1, 9 and 10 are among the highest reported for this type of reaction (Table 1).

Generality. The wide applicability of the present reaction also stands out from the previous ones, where the applicability was generally limited to only one or two combinations of reactants. Not only acylbenzene derivatives (runs 1–11), but also acylheteroaromatics (runs 12 and 13) can be coupled with olefins. A variety of mono- and di-substituted terminal olefins can be employed as shown in Table 1.

Selectivity. When two different reaction sites are available in aromatic ketones and olefins, the regioselectivity of the catalytic reaction is virtually 100%, both for aromatics (runs 8 and 13) and olefins (all runs), producing only one out of the four possible regioisomers. In the case of acetophenone, a 1:2 coupling pro-

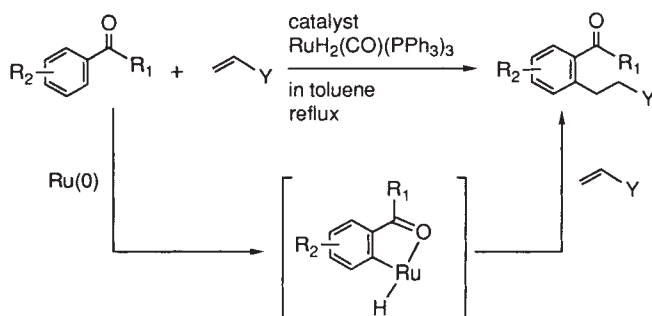
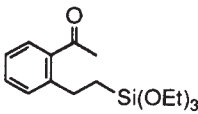
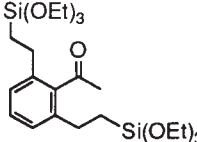
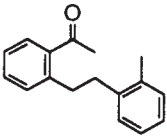
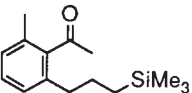
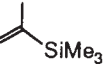
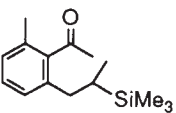
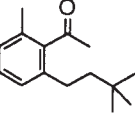
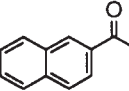
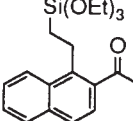
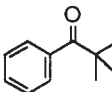
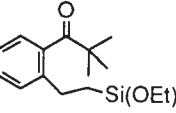
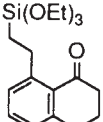
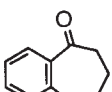
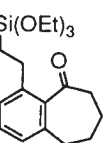
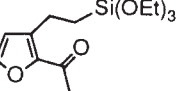
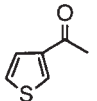
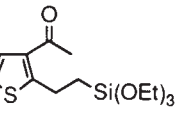


FIG. 1 The Ru-catalysed reaction of aromatic ketones with olefins, showing the cyclometallated intermediate.

TABLE 1 Addition of aromatic ketones to olefins catalysed by $\text{RuH}_2(\text{CO})(\text{P}(\text{C}_6\text{H}_5)_3)_3$

Run	Aromatic ketone	Olefin	Ketone/olefin/catalyst (mmol)	Time (h)	Product	Yield (%)	Product	Yield (%)
1			2 / 2 / 0.04	0.2		75,		8
2			2 / 6 / 0.04	90		<1,		94
3			2 / 4 / 0.04	4		(66)		
4		$\text{CH}_2=\text{CH}_2$ (6 kg cm^{-2})	2 / 12 / 0.04	2		100		
5			2 / 10 / 0.12	4		100		
6			2 / 10 / 0.12	33		96		
7			2 / 10 / 0.04	8		99		
8			2 / 2 / 0.04	6		100		
9			2 / 2 / 0.04	0.5		100		
10			2 / 2 / 0.04	0.5		100		
11			2 / 2 / 0.04	20		88		
12			2 / 2 / 0.04	4		100		
13			2 / 2 / 0.04	1		(90)		

The reactions were run in 3 ml of vigorously refluxing toluene (oil bath temperature 135 °C) as the solvent. For run 4, an autoclave was used. The yields of products were determined by gas chromatography and isolated yields are shown in parentheses. Structures of all products were established unequivocally by standard methods.

duct was obtained in 8% yield, as well as a 1:1 coupling product (75% yield) (run 1). It was possible to prepare the 1:2 product selectively (94% yield) by the use of excess olefin and a prolonged reaction period (run 2).

Possible mechanisms

The mechanism of this new catalytic reaction is not yet clear. The reaction may begin with the coordination of the carbonyl group to the metal, bringing the metal closer to the *ortho* C–H bond. This chelation assistance results in easier, highly site-selective C–H bond cleavage to give the cyclometallated intermediate shown in Fig. 1. Cyclometallation of acetophenone with $\text{CH}_3\text{Mn}(\text{CO})_5$ (ref. 14) and with $\text{RuCl}(\text{OC}(\text{O})\text{CH}_3)(\text{CO})(\text{P}(\text{C}_6\text{H}_5)_3)_3$ (ref. 15) has been known to give products similar to the intermediate postulated in Fig. 1. Acrylic acid esters are known to undergo chelation-assisted oxidative addition to ruthenium complex¹⁷. Insertion of an olefin into the M–H bond thus formed (or into the M–C bond¹⁸), followed by reductive elimination, gives the observed product. The highly selective formation of the 1:1 coupling product in run 9, compared to less

selective results in run 1, is attributed to steric congestion in the 1:1 adduct preventing the carbonyl group from chelating as required for the second olefin coupling. The importance of chelation is further demonstrated by the slower reaction of run 11 compared to run 10; the latter involves a cyclometallated intermediate (Fig. 1) which is almost free from angle strain. Indeed, a corresponding five-membered ketone, 1-indanone (not shown), which should produce greater ring strain in the cyclometallated intermediate, did not react at all.

Applications

This efficient and widely applicable catalytic reaction should open up many opportunities in the field of molecular conversion. The catalytic process described here should make available a new range of alkylated aromatic compounds. Moreover, the approach that we have used—chelation assistance for a metal to facilitate the cleavage of otherwise unreactive bond, followed by reaction of the intermediate so formed with the second reactant—should be applicable more widely for selective organometallic catalysis. □

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Eukaryotic activators function during multiple steps of preinitiation complex assembly

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Eukaryotic activator proteins (activators) stimulate transcription by increasing assembly of the preinitiation complex. We have developed methods to quantify the stable assembly of general transcription factors into transcriptional complexes in response to activators. We show that activators function during at least two stages of preinitiation complex assembly: first, to recruit the general transcription factor TFIIB, and then at a second step, after TFIIB entry. It is at this second step that the TATA-box binding protein associated factors act. This step also seems to be critical for activators to stimulate transcription synergistically.

TRANSCRIPTION initiation by RNA polymerase II involves a stepwise assembly of general transcription factors (GTFs) on the promoter to form a preinitiation complex (PIC) (reviewed in ref. 1; see also Fig. 6). Several studies indicate that transcriptional activator proteins (activators) work, at least in part, by increasing PIC assembly^{2–8}. This implies that one or more steps in PIC assembly are normally limiting, by rate or extent, and are increased by the activator.

We have previously used an immobilized DNA template assay to show that the stable entry of the GTF TFIIB can be a limiting step in complex assembly, and that this step can be enhanced

by an acidic activator⁶. We refer to the increased stable assembly of TFIIB in response to an activator as 'recruitment'. Recruitment could result from an activator-mediated increase in the on-rate of TFIIB binding, a decrease in the off-rate of TFIIB binding, or both⁹. Direct interactions between an acidic activation domain and TFIIB have been demonstrated^{6,10}, and analysis of TFIIB mutants indicates that this interaction is required for transcription activation¹¹. Direct interactions between acidic activators and the TATA-box binding protein (TBP) have also been reported^{12,13}.

The general transcription factor TFIID is composed of TBP

and a set of tightly bound polypeptides, designated TATA-box binding protein associated factors (TAFs). These have 'co-activator' activity: they are required for activator function but do not affect the low level of 'basal' transcription observed in the absence of an activator (reviewed in refs 14–16). Therefore, the TAFs somehow enable an activator to increase PIC assembly.

We have previously used transcription complementation assays to deduce which GTFs were stably bound to the promoter in response to an activator⁶. The reliance on transcription activity restricted several of our conclusions. For example, it has been proposed that activators function by preventing transcriptional complexes from entering a non-productive pathway (see, for example, refs 8, 17). According to these models, the activator may not affect the amount of a bound GTF, but rather only increase the percentage of functional complexes. These two possibilities could not be distinguished by assaying only transcription activity. Furthermore, a transcription assay can identify only the first step that limits PIC assembly; a possible downstream limiting event would not be detected.

We have developed new methods to purify activated transcription complexes and measure stably incorporated GTFs directly. Here we use these methods to address the following questions. First, is the sole function of an activator to recruit TFIIB to the promoter? Second, at which step(s) of PIC assembly do TAFs function? Third, where during PIC assembly does activator cooperativity first become evident? Fourth, do acidic and non-acidic activators have similar or different effects on PIC assembly?

Our results confirm that activators increase stable assembly of TFIIB. In addition, we find that activators must affect a subsequent step to stimulate transcription.

Experimental design

To extend our previous study⁶, we have developed methods to detect directly GTFs stably incorporated into transcriptional complexes. In the experiments presented below, the activators were GAL4 derivatives containing either of two well-characterized acidic activation regions (GAL4-AH, ref. 18 or GAL4-VP16, ref. 19), or a non-acidic activation domain (GAL4-Gln, ref. 20; or GAL4-Pro, ref. 21). GTFs stably incorporated into transcriptional complexes were purified away from unbound GTFs by gel-filtration chromatography or by a modified immobilized DNA template system (see below). The amount of stably assembled GTFs, measured by immunoblotting, revealed the extent of PIC assembly.

Preliminary experiments revealed that in our previously described immobilized DNA template assay⁶, GTFs bound non-specifically to the streptavidin-agarose beads, resulting in an unacceptably high background in an immunoblot assay (data not shown). We developed two experimental strategies to circumvent this problem. First, we used gel-filtration chromatography to purify PICs. A similar approach has been used to purify RNA polymerase III transcription complexes²². In Fig. 1a a transcription reaction mixture was fractionated on a Sepharose CL2B column. The DNA template and transcription activity co-eluted, with a peak in fractions 10 and 11 (Fig. 1a). In the absence of an activator, the column profile was qualitatively identical but, as expected, the level of transcription from the purified complexes was much lower (data not shown). Most of the proteins in the nuclear extract, including the GTFs, were distributed in fractions 13–48 (data not shown and see below). Thus, gel-exclusion chromatography can be used to separate transcription complexes from unbound nuclear extract proteins.

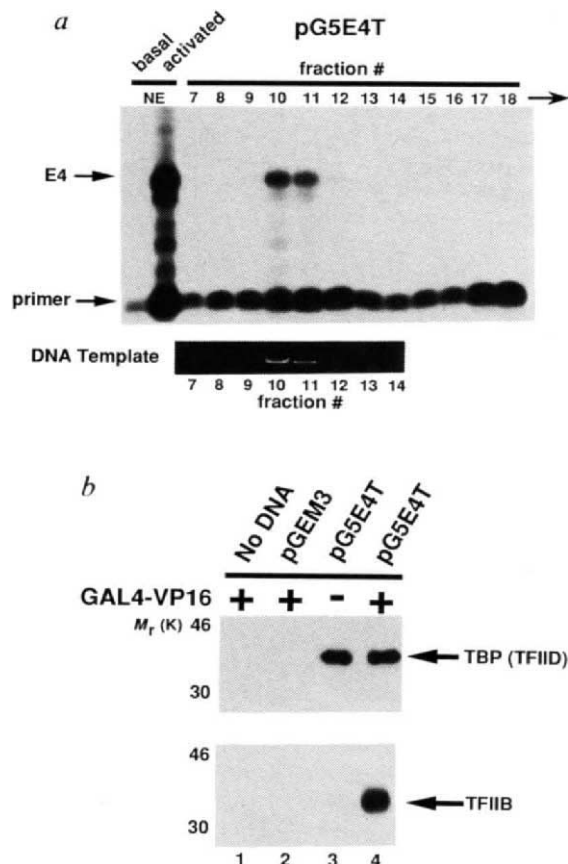


FIG. 1 An acidic activator increases stable assembly of TFIIB, but not TFIID. **a**, Sepharose CL2B gel filtration column profile. A 200- μ l reaction mixture containing nuclear extract, GAL4-VP16, and the DNA template pG5E4T was fractionated on a Sepharose CL2B column; each fraction was analysed for transcription activity (top panel) and for the presence of DNA template (bottom panel). NE, HeLa cell nuclear extract. The position of the primer and the primer extension products representing accurately initiated RNA (E4) are shown on the left. **b**, Complex assembly assay. Reaction mixtures containing nuclear extract and the indicated DNA template were fractionated on a Sepharose CL2B column, and the major DNA containing fractions (9–12) were pooled and analysed by immunoblotting for either TBP (top panel) or TFIIB (bottom panel). The presence (+) or absence (–) of the activator is indicated above each lane. The positions of relative molecular mass (M_r) standards are indicated on the left.

METHODS. A 1 $\times$ 30-cm gravity flow column was packed with Sepharose CL2B pre-equilibrated with 12.5 mM HEPES pH 8.0, 7.5 mM MgCl₂, 0.125 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride (PMSF). Reaction mixtures (200 μ l) contained 125 μ l nuclear extract, 1 μ g DNA template (pG5E4T) and 10 μ g pGEM3 DNA digested with *Hae*III and *Hpa*II. After 30 min incubation at 30 °C, the sample was loaded on the column, eluted with transcription buffer (70 mM KCl, 20 mM Tris-HCl pH 8.0, 7.5 mM MgCl₂, 0.3 mM EDTA, 0.3 mM PMSF) and 48 300- μ l fractions were collected. Transcription was initiated by addition of ribonucleoside triphosphates to a final concentration of 625 μ M and 40 U RNasin followed by incubation for 30 min at 30 °C. Transcripts were detected by primer extension⁶. For analysis of complex assembly, the major DNA-containing fractions (9–12) were pooled and precipitated with 10% trichloroacetic acid (TCA)/0.01% deoxycholate. The pellet was rinsed with acetone, dried and resuspended in SDS-PAGE sample buffer. Polypeptides were fractionated on either a 12.5% or an 8% SDS-PAGE gel and transferred to a Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in a mixture containing 5% milk powder and 150 mM NaCl, 25 mM Tris-HCl (pH 7.6), 0.1% Tween-20 (TBS-Tween-20). The TBP and TFIIB antibodies were described in ref. 10. The secondary antibody was either horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Jackson Immunoresearch Labs) or HRP-conjugated anti-mouse IgG (Jackson Immunoresearch Labs). The membranes were developed with Amersham ECL detection kits and exposed to Amersham MP film.

Second, we have modified the original immobilized DNA template assay^{6,23} by using streptavidin coupled to metal beads (Dynabeads M-280), rather than streptavidin-agarose, which minimizes nonspecific binding of GTFs (see below).

An acidic activator increases TFIIB assembly

To determine how activators increase PIC assembly, complexes were assembled in the presence or absence of an activator, and DNA-bound GTFs were quantified by immunoblot analysis of the major DNA-containing fractions. We previously demonstrated that the acidic activator GAL4-AH recruited TFIIB to the promoter⁶. It was important to determine whether a different acidic activator had a comparable effect and therefore in this experiment we used the acidic activator GAL4-VP16 (ref. 19). In Fig. 1*b*, complexes were assembled in nuclear extract in the presence and absence of GAL4-VP16 and analysed for two

GTFs, TFIID and TFIIB. An anti-TBP antibody was used to monitor the TFIID complex. When the crude nuclear extract was incubated in the absence of a DNA template (Fig. 1*a*, 'No DNA') or with DNA lacking a promoter ('pGEM3'), there was no detectable TBP or TFIIB in complexes (Fig. 1*a*, lanes 1, 2). After addition of an adenovirus (Ad) E4 promoter derivative (pG5E4T), TBP was now found associated with the DNA template (Fig. 1*a*, lane 3). Significantly, the amount of DNA-bound TBP was equivalent in the presence or absence of GAL4-VP16 (Fig. 1*a*, compare lanes 3 and 4). In contrast, GAL4-VP16 substantially increased the amount of TFIIB in complexes (Fig. 1*b*, compare lanes 3 and 4) in a manner that paralleled transcriptional activity (Fig. 1*a*, lanes 1, 2). Thus, on the Ad E4 promoter, which contains a consensus TATA box, both GAL4-VP16 (Fig. 1*b*) and GAL4-AH (ref. 6 and see below) increased the stable assembly of TFIIB, but not that of TFIID. These conclusions are identical to those of our previous study using immobilized DNA templates, a different acidic activator (GAL4-AH) and an assay involving biochemical complementation of transcription activity⁶.

Both TFIID and TBP support TFIIB recruitment

Our previous study suggested that an acidic activator could recruit TFIIB only if TFIID was also present⁶. Because TAFs are required for activated transcription (reviewed in refs 14–16), we compared TFIID and TBP for their ability to support TFIIB recruitment. In Fig. 2*a* and *b*, the reaction mixtures contained either recombinant TBP or HeLa TFIID, and all other GTFs. As expected from previous studies, the TFIID complex, but not TBP, supported activated transcription (Fig. 2*a*, compare lanes 5 and 6). Strikingly, however, Fig. 2*b* shows that the acidic activator GAL4-AH recruited TFIIB when either TBP (lane 3) or TFIID (lane 4) was present.

To confirm and extend this conclusion, we analysed TFIIB assembly using purified proteins in the absence of extract. In Fig. 2*c* the reaction mixtures contained combinations of three purified recombinant proteins, GAL4-AH, TBP and TFIIB. When either TBP or GAL4-AH was bound to the promoter, a small amount of TFIIB was assembled into complexes (Fig. 2*c*, compare lanes 2 and 3 with lane 1). These data are consistent with the findings that TFIIB interacts directly with both TBP²⁴ and an acidic activation domain^{10,11}. Most importantly, the amount of TFIIB assembled in the presence of both GAL4-AH and TBP (Fig. 2*c*, lane 4) was much greater than when either DNA-binding protein was present alone (lanes 2 and 3). We conclude that TBP can support the activator-mediated recruitment of TFIIB.

Using native gels to assay complex assembly, we have found that an acidic activator increased TFIIB binding approximately 10-fold in the presence of TBP (data not shown). This increase in TFIIB assembly was, however, less than that observed in the gel-filtration and immobilized DNA template assays (see, for example, Fig. 2). A likely explanation for this quantitative difference is that the gel-filtration and immobilized DNA template assays demand that a GTF remain stably associated with the DNA for a substantial time; in contrast, native gels may 'trap' relatively unstable intermediates (for example, the TFIIB–TBP–DNA complex). In this regard, PICs and intermediates formed in the absence of an activator have been previously detected using native gel assays¹. However, these previous native gel experiments, which used TBP rather than TFIID, could not address whether the activator increased PIC assembly.

Recruitment of GTFs that enter after TFIIB

Eukaryotic activators increase PIC assembly^{2–8}. The fact that TBP cannot support activated transcription implies that in the absence of TAFs, PIC assembly stalls at a specific step. Figure 2 demonstrated that TBP can support activation through the TFIIB-binding step, implying that assembly arrests subsequently.

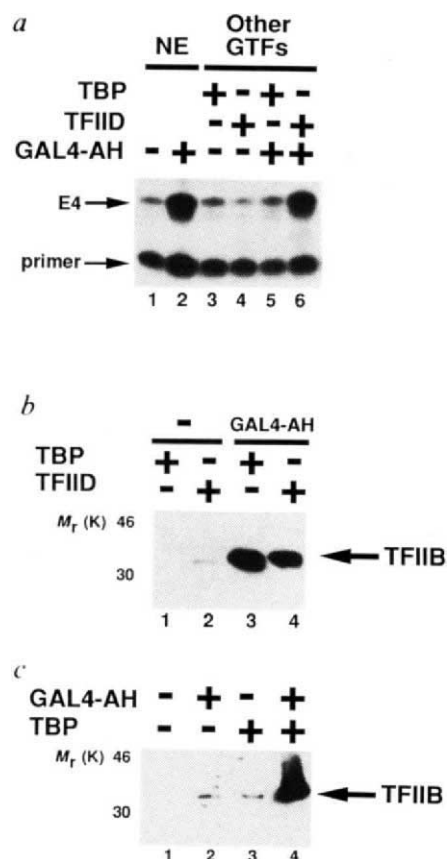


FIG. 2 Both TFIID and TBP can support the activator-mediated recruitment of TFIIB. *a*, Transcription activity in a reconstituted system. A TFIID-depleted extract (other GTFs) was incubated with the DNA template pG5E4T and the indicated factors. *b*, TFIIB assembly. Reaction mixtures contained the TFIID-depleted fraction (other GTFs), pG5E4T and the indicated factors. The reaction mixtures were fractionated on a gel-filtration column and the DNA-containing fractions analysed by immunoblotting for TFIIB. *c*, Assembly of TFIIB with three purified proteins. As in *b*, except that the reaction mixtures contained only the indicated purified recombinant proteins.

METHODS. As a convenient source of GTFs lacking TFIID, we found that the S100 fraction resulting from the preparation of a nuclear extract typically contained all GTFs, but little or no TFIID activity. Any residual TFIID activity was removed by chromatography on a P11 column. A 0.6 M KCl eluate was collected and dialysed against 0.1 M KCl, 20 mM HEPES, 0.5 mM EDTA, 0.5 mM PMSF, 20% glycerol and tested for transcription in the absence and presence of added TFIID; the fraction was used only if it was transcriptionally incompetent alone and if addition of TFIID restored full activity. Purification of HeLa TFIID (ref. 6) and recombinant TBP (ref. 47) has been described previously.

To identify this step, complexes assembled in the presence of either TBP or TFIID were purified by gel-exclusion chromatography and analysed using antibodies to three GTFs that enter the complex after TFIIB: the 30K (relative molecular mass 30,000) subunit of TFIIF (TFIIF³⁰), the 56K subunit of TFIIE (TFIIE⁵⁶) or the largest subunit of RNA polymerase II. These data are shown in Fig. 3a and allow us to derive several conclusions. First, as with TFIIB, the acidic activator substantially increased the stable assembly of TFIIF³⁰, TFIIE⁵⁶ and RNA polymerase II (Fig. 3a, compare lanes 2 and 6). This increase did not occur with GAL4(1-94), which lacks an activation domain (Fig. 3a, compare lanes 4 and 6). These data are consistent with previous studies which used transcription assays to show that activators increase PIC assembly²⁻⁸. Second, and most importantly, in the presence of TBP, the activator failed to recruit TFIIF³⁰, TFIIE⁵⁶ or RNA polymerase II (Fig. 3a, compare lanes 5 and 6). Thus, TBP can support the activator-mediated recruitment of TFIIB (Fig. 2), but in the absence of TAFs these complexes fail to progress to functional PICs.

The interpretation of these combined results is that recruitment of TFIIB is not sufficient for transcriptional activation. A

prediction of this idea is that an increased concentration of TFIIB will not, by itself, overcome the requirement for an activator. Increasing the concentration of TFIIB 1,000-fold did not circumvent the requirement for the activator nor diminish its effect (Fig. 3b).

Activator cooperativity exerted after TFIIB entry

Multiply-bound activators can stimulate transcription synergistically (reviewed in ref. 25) and this effect is exerted after binding of the activator to DNA^{26,27}. We sought to identify the step of assembly at which activator cooperativity is first observed. Figure 4a shows that, in a nuclear extract, a single bound activator stimulated transcription only twofold, whereas five bound activators stimulated transcription approximately 50-fold. A single bound activator substantially increased the amount of assembled TFIIB (Fig. 4b, compare lane 7 with 3 and 5), whereas five bound activators further increased TFIIB assembly approximately twofold (compare lanes 7 and 8). Thus, multiple activators do not have a synergistic effect on TFIIB recruitment. In contrast, there was a large, synergistic increase in the amount of TFIIE⁵⁶ stably assembled by five compared with one bound activator (Fig. 4b, compare lanes 7 and 8), corresponding to the transcriptional increase (Fig. 4a). Results similar to those observed with TFIIE⁵⁶ were obtained using antibodies directed

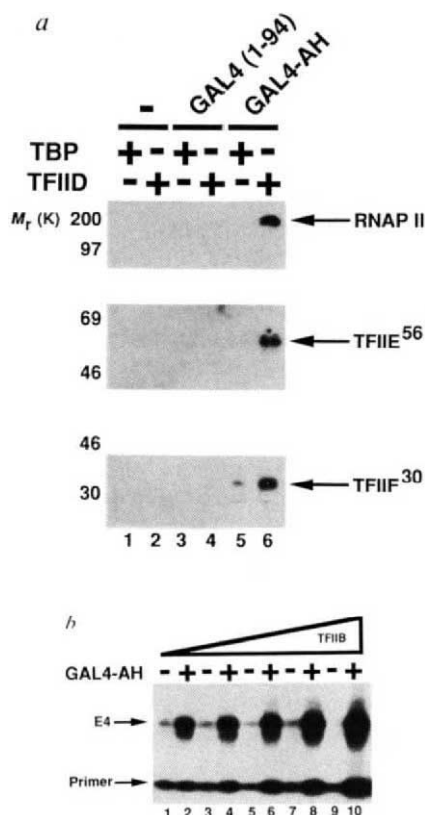


FIG. 3 TFIID, but not TBP, can support the activator-mediated recruitment of GTFs that assemble after TFIIB. *a*, Assembly of TFIIF, TFIIE and RNA polymerase II. Reaction mixtures were prepared as described for Fig. 2a and contained the indicated GAL4 derivative. Purified complexes were analysed by immunoblotting for the indicated GTF. *b*, TFIIB titration. Various amounts of purified recombinant TFIIB were added to a TFIIB-depleted HeLa nuclear extract and assayed for transcription from the DNA template pG5E4T in the absence (–) and presence (+) of the activator, GAL4-AH. The amounts of recombinant TFIIB added were: lanes 1, 2, 0.4 ng; lanes 3, 4, 2 ng; lanes 5, 6, 4 ng; lanes 7, 8, 20 ng; lanes 9, 10, 400 ng.

METHODS. Antibodies to TFIIF³⁰ (ref. 48) and TFIIE⁵⁶ (ref. 49) were a gift of D. Reinberg. A monoclonal antibody to RNA polymerase II large subunit⁵⁰ was a gift of N. Thompson. Preparation of TFIIB-depleted nuclear extract and recombinant TFIIB was performed as described previously^{6,10,11}.

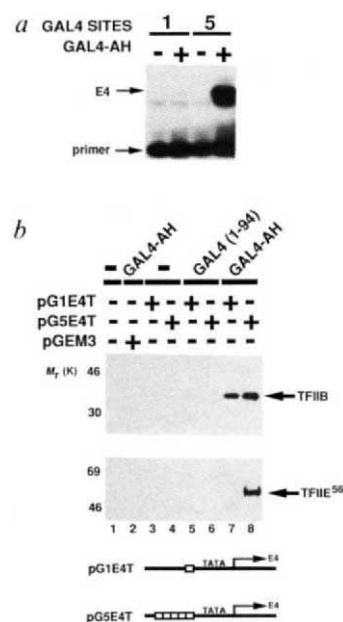


FIG. 4 Effect of multiple activators on complex assembly. *a*, Transcription activity. Reaction mixtures contained nuclear extract and an immobilized DNA template bearing either one (pG1E4T) or five (pG5E4T) GAL4-binding sites. A diagram of the DNA templates is shown at the bottom. GAL4-binding sites are indicated by boxes. *b*, Complex assembly. Reaction mixtures were as in *a* and contained the indicated GAL4 derivative and DNA template. Complexes were purified on immobilized DNA templates and analysed by immunoblotting for either TFIIB (top panel) or TFIIE⁵⁶ (bottom panel).

METHODS. The EcoRI/PvuII fragment of the appropriate DNA template was 3' end-labelled with biotin-14-dATP (BRL) using AMV reverse transcriptase. Dynabeads M-280 streptavidin (Dyna) were prewashed twice in phosphate-buffered saline/0.1% BSA. Dynabeads (60 µg per µg DNA) were incubated on ice for 1 h in transcription buffer. The bound DNA was then purified using a magnetic particle concentrator (Dyna). PICs were formed on biotinylated DNA templates using 3 µl of beads (500 ng DNA) per 40-µl reaction volume. Complexes were magnetically purified, and the beads washed three times in transcription buffer containing 0.003% Nonidet P40. SDS sample loading buffer was added and polypeptides resolved by SDS-PAGE.

against other GTFs that assemble following TFIIB (data not shown). We conclude that multiple activators increase PIC assembly synergistically and that this increase occurs at a step following TFIIB entry.

Non-acidic activators

The results described above and in our previous studies were performed with acidic activators. If TFIIB recruitment is necessary for transcription activation, then non-acidic activators must also recruit TFIIB. To test this prediction, we analysed two well-characterized non-acidic activators: GAL4-Gln, which contains a glutamine-rich activation region²⁰, and GAL4-Pro, which contains a proline-rich activation region²¹.

The transcription data of Fig. 5a demonstrate that, like GAL4-AH, both GAL4-Gln and GAL4-Pro efficiently activated transcription in a nuclear extract. In Fig. 5b, complexes were assembled in a nuclear extract, purified and analysed for TFIIB and TFIIE. The results show that, like acidic activators, the two non-acidic activators substantially increased assembly of both TFIIB and TFIIE. These results provide additional support for the notion that recruitment of TFIIB is necessary for transcriptional activation.

Discussion

Based on the results presented here and in previous studies, PIC assembly can be divided into at least three stages (Fig. 6). First, the two sequence-specific DNA-binding proteins—the activator and the GTF TFIID—bind to their respective sites on the DNA template. Second, the bound activator makes contact with TFIIB, thereby recruiting it to the promoter. Third, a second activator-dependent step, which involves TAFs, is required for activated transcription.

When a promoter that has a consensus TATA box is used, we have detected no effect of an activator on TFIID binding (ref. 6 and this report). It remains possible, however, that on other promoters, in particular those with non-consensus TATA boxes, an activator may increase TFIID binding. Indeed, in

other systems both quantitative²⁸ and qualitative^{29,30} effects of an activator on TFIID binding have been reported. However, as discussed further below, even in these instances the major consequence of an activator-TFIID interaction could be at a later stage of PIC assembly.

TFIIB recruitment

The results presented here and in other studies suggest that the activator-mediated recruitment of TFIIB is a critical step in the activation process. Activators function by increasing PIC assembly²⁻⁸, which entails increasing the amount of specific intermediates in the assembly pathway. In assays that monitor PIC assembly, TFIIB binding can be a limiting step, which is enhanced by two unrelated acidic activators (ref. 6 and this report). Here we have shown that two non-acidic activators also recruit TFIIB. The fact that all of these activators recruit TFIIB argues strongly for the functional relevance of this event. Similarly, kinetic assays indicate that TFIIB binding is a slow step, which can be accelerated by an activator³¹.

Recruitment of TFIIB by an acidic activator can occur in the crude nuclear extract and with purified components, and in the presence or absence of downstream GTFs. These observations suggest that recruitment is due to a direct acidic activator-TFIIB interaction. Indeed, both HeLa TFIIB^{6,10} and recombinant TFIIB^{10,11} interact with an acidic activation region. Interactions between TFIIB and several non-acidic activators have also been reported^{32,33}. Finally, TFIIB mutants unable to interact with the VP16 acidic activation region are specifically defective in supporting transcription directed by acidic activators¹¹. Conversely, a VP16 single amino-acid substitution mutant, which cannot activate transcription³⁴, is correspondingly defective for interaction with TFIIB^{6,10}.

From the results presented here and our previous work, we propose a physical basis for TFIIB recruitment (Fig. 6). Protein-protein interaction studies have shown that TFIIB can interact with both TBP²⁴ and an acidic activator^{10,11}. When bound on the promoter, the activator and TBP are brought into proximity. We propose that TFIIB binds simultaneously to the two adjacent promoter-bound proteins. Such simultaneous contact would pre-

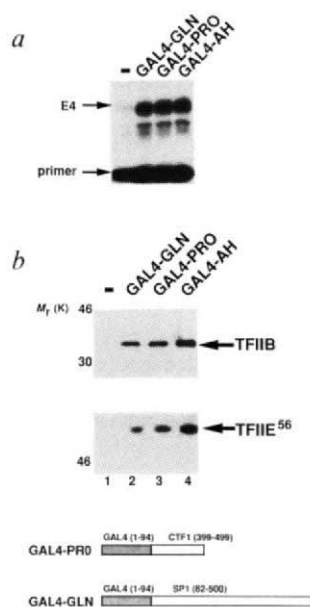


FIG. 5 Effect of non-acidic activators on complex assembly. *a*, Transcription activity. Reaction mixtures contained nuclear extract, the DNA template pG5E4T and the indicated GAL4 derivative. A schematic representation of the two non-acidic activators is shown at the bottom. *b*, Complex assembly. Reaction mixtures were as in *a*. Complexes were purified and analysed by immunoblotting for either TFIIB (top panel) or TFIIE⁵⁶ (bottom panel).

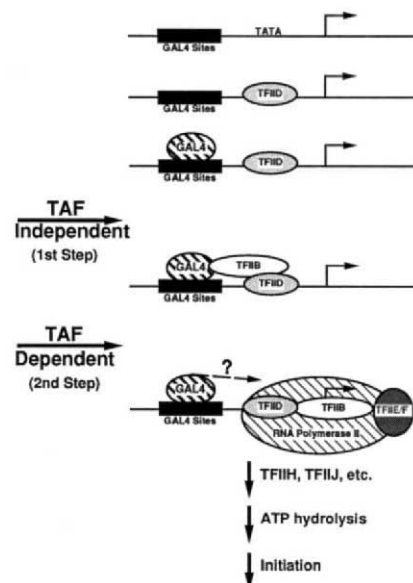


FIG. 6 Model for activator-mediated assembly of preinitiation complexes. A simplified version of PIC assembly is shown; not all GTFs are drawn in the complex, and only one of the multiple bound activators is indicated. Some of the protein-protein and protein-DNA contacts are drawn arbitrarily. The question mark indicates that for the second step the relevant target of the activator is not known.

dict that TFIIB would bind cooperatively, which is consistent with the data of Fig. 2c. Because TFIIB interacts directly with the TBP subunit of TFIID, both TFIID and TBP can support recruitment by the activator.

We have shown that two non-acidic activators behave identically to an acidic activator in recruiting both TFIIB and downstream GTFs to the promoter. Although these non-acidic activators can recruit TFIIB in a crude nuclear extract, we do not yet know if this is due to a direct activator-TFIIB interaction or whether it is mediated through intermediary proteins, perhaps TAFs. In support of this latter possibility, we have not detected a direct interaction between TFIIB and a non-acidic activator by the same methods used to characterize an acidic activator-TFIIB interaction (data not shown).

The second step

We have provided three lines of evidence that TFIIB recruitment is not sufficient for transcriptional activation. First, TBP can support recruitment of TFIIB (Fig. 2), but not of downstream GTFs (Fig. 3a). The failure to recruit downstream GTFs, including RNA polymerase II, explains why transcription is not activated in the absence of TAFs. Second, raising the concentration of TFIIB does not overcome the requirement for an activator (Fig. 3b). Third, whereas a single bound activator can recruit TFIIB efficiently (Fig. 4), high levels of transcription are not achieved.

These results implicate a second step, occurring after TFIIB entry, that is affected by activators. The notion that activators may affect several steps would explain why raising the concentration of a single GTF does not overcome the requirement for an activator (Fig. 3b and refs 35, 36). During this second step, the activator could act directly by making contact with a GTF (or a co-activator) that assembles after TFIIB. Alternatively, the activator could affect this second step indirectly by working through GTFs that assemble early, such as TFIID and TFIIB. For example, the activator may induce a conformational change in TFIIB that facilitates assembly of downstream GTFs.

We have shown that one or more TAFs is essential for this second step. It has been speculated that TAFs are direct targets of various activation regions¹⁴, and an interaction between a glutamine-rich activation region and a particular TAF, TAF-110, has been reported³⁷. Although the functional significance of this interaction remains to be proven, this finding is not inconsistent with our results: the major consequence of an activator-TAF interaction could occur at a late stage of PIC assembly.

It is interesting that in the absence of TAFs, the activator increases the amount of the TBP-TFIIB complex without affecting transcription (Fig. 2). One explanation for this is that the activator-TBP-TFIIB complex is non-functional in the absence of TAFs. Co-activator activities that seem to be distinct from TAFs have also been described³⁸⁻⁴², and their role in the assembly pathway remains to be determined.

Activator cooperativity

Our experiments on transcriptional synergy also point to an essential step following TFIIB entry: a single activator recruits TFIIB, but high levels of transcription are not achieved because downstream GTFs do not assemble efficiently. Multiple activators increase recruitment of downstream GTFs synergistically. Our results confirm and extend those of Wang *et al.*⁷, who used a chemical footprinting assay to conclude that multiple activators increase the number of transcription complexes.

To help explain transcriptional synergy (reviewed in ref. 25), we have previously suggested that the multiple promoter-bound activators may form contacts with more than one transcription component^{6,10,11}. For example, one bound activator could make contact with TFIIB and recruit it into the complex and another bound activator could make contact with another GTF (or a co-activator). In support of this idea, interactions between activators and factors other than TFIIB, such as TBP^{12,13,28,43-46} and TAFs³⁷, have been reported. From these considerations, we suggest that eukaryotic activators interact with multiple targets to affect several steps of PIC assembly. □

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Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane

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& Pär Nordlund^{§‡}

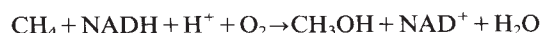
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The 2.2 Å crystal structure of the 251K $\alpha_2\beta_2\gamma_2$ dimeric hydroxylase protein of methane monooxygenase from *Methylococcus capsulatus* (Bath) reveals the geometry of the catalytic di-iron core. The two iron atoms are bridged by exogenous hydroxide and acetate ligands and further coordinated by four glutamate residues, two histidine residues and a water molecule. The dinuclear iron centre lies in a hydrophobic active-site cavity for binding methane. An extended canyon runs between $\alpha\beta$ pairs, which have many long α -helices, for possible docking of the reductase and coupling proteins required for catalysis.

METHANOTROPHIC bacteria play an essential part in cycling carbon in the biosphere by consuming methane produced in anaerobic sediments and by limiting its flux to the atmosphere where it acts as a greenhouse gas^{1,2}. The first step in this process is catalysed by methane monooxygenase (MMO) systems, which convert methane to methanol:



The soluble MMO in several methanotrophs consists of three proteins, a hydroxylase ($M_r \sim 251\text{K}$), a reductase (38.6K), and a coupling protein (15.5K), all three of which are required for enzymatic activity³. The hydroxylase is comprised of three polypeptides (α , 60.6K; β , 45.0K; γ , 19.8K) of $\alpha_2\beta_2\gamma_2$ stoichiometry; it contains a dinuclear iron centre^{4–6} responsible for methane hydroxylation and belongs to a growing class of functionally diverse non-haem di-iron proteins^{7–10}. As isolated, the di-iron centre in the MMO hydroxylase is in the fully oxidized, Fe(III)Fe(III), state; the reductase transfers electrons from NADH to the hydroxylase¹¹, and the coupling protein¹² appears to have several regulatory activities.

Apart from their biological functions and ecological importance, the unique ability of MMOs to oxidize a broad range of hydrocarbons in addition to methane¹³ has attracted attention. This property has led to several applications of methanotrophic bacteria, including bioremediation of land contaminated by oil spills^{14,15} and the oxidative removal of trichloroethylene from drinking water^{16,17}. At present methane is underutilized as an energy source owing to the lack of efficient processes for its conversion to liquid form (as methanol, for example) to facilitate transport¹⁸. A new hydroxylation catalyst based on an understanding of the MMO system would make more efficient use of the world supply of natural gas.

Until now, the available structural information about the hydroxylase and its catalytic di-iron centre was derived from spectroscopic studies of the enzymes isolated from *M. capsulatus* (Bath)^{4,6,19,20} and *M. trichosporium* OB3b^{6,21,22}, combined with various sequence alignments with other non-haem-iron proteins^{10,23,24}. Here we present the X-ray crystal structure at 2.2 Å resolution of the *M. capsulatus* (Bath) MMO hydroxylase.

The three-dimensional structure of this enzyme provides important insight into biological methane oxidation, including how this inert gas might diffuse to and bind near the active site, the identification of protein residues that could participate in the catalytic mechanism, possible domains of interaction with the coupling protein and reductase, and the architecture of the dinuclear iron core.

The hydroxylase structure

The structure of the $\alpha_2\beta_2\gamma_2$ dimer was solved by standard crystallographic methods and refined to a current R -value of 0.170 at 2.2 Å resolution with good stereochemistry (Table 1). The enzyme is a relatively flat molecule with approximate dimensions $60 \times 100 \times 120$ Å. The arrangement of the subunits, two $\alpha\beta\gamma$ protomers related by a non-crystallographic twofold symmetry axis, is shown in Fig. 1. There is a wide canyon running along the dimer interface with an opening in the centre of the molecule. The secondary structure of the three subunits (Fig. 2) is primarily helical, with one small region of β -structure in the α -subunit. This subunit contains two domains, one of which is comprised of six very long helices (A–C and E–G) and six smaller ones. This N-terminal domain is arranged in three layers, the first containing helices A–D and 1–2, the second composed of helices E–G and 3, and the third containing helices H and 4. The second domain, at the carboxy terminus, consists of seven smaller helices and two β -hairpin motifs. The β -subunit contains 12 helices, six of which are 25 residues or longer. A remarkable feature of the structure is that ten helices of the α - and β -subunits possess virtually identical folds. The two subunits have completely different primary sequences, however, with no detectable homology. A very similar fold is also seen in the R2 protein of ribonucleotide reductase (Fig. 1)^{25,26}. The γ -subunit, which lies on the outer edges of the molecule at the interface of the α - and β -subunits, consists of eight helices (Fig. 2).

The interaction between the two protomers involves helices from the α - and β -subunits, with no participation of the two γ -components (Fig. 1). Most of the dimer contacts involve the β -subunits. Interactions between α - and β -subunits within the protomers resemble those in the ribonucleotide reductase R2 protein dimer interaction, which has the shape of a heart^{25,26}. The hydroxylase dimer can therefore be viewed as the juxtaposi-

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tion of two heart-shaped protomers to form a molecule of more than twice the size (Fig. 1).

The dinuclear iron centres are positioned entirely within the α -subunits, which is consistent with chemical labelling²⁷, and appear to be fully occupied according to the current difference Fourier ($F_o - F_c$) maps and refinement. Thus the *M. capsulatus* hydroxylase can accommodate four iron atoms, although the purified enzyme typically contains nearer to two iron atoms per protein molecule⁶. One explanation for this result is that we have selectively crystallized hydroxylase containing four iron atoms from a mixture having some apo (iron-free) and/or iron-depleted protein. This finding supports quantitative work on the *M. trichosporium* hydroxylase, which shows that there are two di-iron centres²⁸, but contradicts reports that the *M. capsulatus* hydroxylase contains only a single di-iron site²⁹. The two dinuclear iron centres are 45 Å apart. The iron ligands are derived from helices B, C, E and F, which form a four-helix bundle, in accord with the model suggested by comparison of the ribonucleotide reductase R2 protein and hydroxylase α -subunit gene sequences²⁴. The iron atoms are buried below helices E and F, which together form one wall of the canyon.

Coordination of the di-iron centre

The two dinuclear iron centres were refined independently and their coordination spheres and geometries agree well in most

respects. The $2F_o - F_c$ electron density map at one of the di-iron centres is shown in Fig. 3; a schematic diagram of the active-site geometry is presented in Fig. 4. The Fe...Fe distances in the two protomers are both 3.4 Å, consistent with the 3.42 Å value determined by EXAFS spectroscopy⁶. The pseudo-octahedral iron atoms are linked by two non-protein bridging ligands, which were apparent in difference ($F_o - F_c$) Fourier maps during the refinement. We have modelled these two exogenous ligands as monodentate hydroxide and bidentate acetate ions. We considered that the bidentate ligand might be bicarbonate ion, but prefer acetate because there are no hydrogen-bonding interactions with the non-coordinated portion of the ion and because we had 50 mM ammonium acetate in the crystallization buffer. Although the nature of the monodentate bridging ligand in the di-iron(III) form of the hydroxylase has not been elucidated by spectroscopy, the presence of a hydroxo bridge in the mixed valent, Fe(II)Fe(III) form has been revealed by proton ENDOR studies^{20,22}. It is conceivable that in cells the resting enzyme might contain a bridging acetate or bicarbonate ligand, but such a bridge would be lost on reduction of the di-iron centre during catalysis. The space occupied by the exogenous acetate ligand in the present crystal structure determination might demarcate where the oxidized substrate, methoxide ion, normally resides but before its protonation and release from the active site. The binding of exogenous ligands such as azide, fluoride³⁰ and

TABLE 1 Data collection, phasing and refinement statistics

Compound	Resolution (Å)	Unique reflections	Completeness (%)	R_{merge} (%) [*]	Isomorphous difference (%)	Number of sites	Phasing power [†] (resolution)
Native	2.2	103,751	92	9.4			
PIP1 [‡]	3.7	12,283	52	4.0	18.7	9	1.83 (4.3 Å)
PIP2	3.7	7,454	31	3.7	18.6	9	1.77 (3.8 Å)
TAMM1	3.0	18,147	41	7.0	19.2	11	1.17 (3.5 Å)
TAMM2	3.0	18,279	41	7.9	19.8	9	1.32 (3.8 Å)
TAMM3	3.0	18,597	42	6.4	19.0	11	1.23 (3.5 Å)
TAMM4	3.0	15,215	34	6.7	19.1	11	1.32 (3.5 Å)
EMTS1	3.0	18,012	40	7.5	14.8	9	1.55 (3.5 Å)
EMTS2	3.0	27,471	62	6.3	15.0	9	1.78 (3.5 Å)
EMTS3	3.0	18,634	42	5.1	15.0	9	1.53 (3.5 Å)

Crystals: Crystals of the hydroxylase were obtained by using 20% PEG 4000, 25 mM Li₂SO₄, 50 mM NH₄OAc in 25 mM MOPS, pH 7.0 (ref. 45). The hydroxylase was purified as described previously⁶. The space group is orthorhombic P2₁2₁2₁ with unit cell dimensions $a = 62.6$ Å, $b = 110.1$ Å, $c = 333.5$ Å, and the asymmetric unit contains one $\alpha_2\beta_2\gamma_2$ dimer. Diffraction data: The native data set used for the structure determination was obtained at the Photon Factory, Tsukuba, Japan, using a modified Weissenberg camera and imaging plates. Diffraction intensities were measured using the program WEIS. Heavy-atom derivative data were collected at Stanford Synchrotron Radiation Laboratory by using a Marresarch imaging plate detector, and were processed with the program DENZO (written by Z. Otwinowski). All data were merged and scaled by using programs in the CCP4 program suite. The difference Patterson map for the PIP derivative was solved with the use of the program RSPS⁴⁶, and several strong sites were identified and refined with the programs HEAVY⁴⁷ and MLPHARE⁴⁸. The heavy-atom structures of the other derivatives were determined from difference Fourier maps phased by the PIP derivative. Three types of derivatives were used in the final phasing and the mean figure of merit was 0.47 (MLPHARE) to 3.5 Å resolution. The anomalous data were not of sufficient quality to be used in the phasing. The MIR phases were improved and extended by solvent flattening⁴⁹ and twofold molecular averaging using RAVE⁵⁰. Non-crystallographic symmetry: The non-crystallographic twofold axis relating the two halves of the $\alpha_2\beta_2\gamma_2$ dimer was located by using the positions of the heavy atoms, eight pairs of which were used to refine the symmetry matrix. The heavy atom parameters were then refined using the density-modified phases as fixed protein phases^{51,52}, and the new MIR phases were used to improve the molecular envelope and twofold symmetry matrix. Model building and refinement: Skeletonized electron density maps⁵³ were used to identify the subunits and to locate secondary structure. The program O was used for all model building⁵⁴. Initially, the sequence⁵⁵ of the iron-coordinating four-helix bundle in the α -subunit was modelled into the electron density based on the presence of two mercury binding sites, which were postulated correctly to be the cysteine residues near the di-iron centre (Cys 151 and Cys 211) predicted by the sequence alignment with the ribonucleotide reductase R2 protein²⁴. In addition, a number of long helices were built as polyaniline chains. This first model was subjected to simulated annealing refinement by using X-PLOR⁵⁶, and partial model phases were combined with averaged phases, resulting in a slightly improved map. A large number (~30) of iterations of model building, refinement and phase combination were performed. The sequences of the β - and γ -subunits were identified using several of the heavy-atom sites. The current model containing 512 of the 527 residues in the α -subunit, 384 of the 389 residues in the β -subunit and 162 of the 170 residues in the γ -subunit was obtained. The electron density maps of the C terminus of the β -subunit revealed an error in the published sequence⁵⁵ (residue 363 is a Trp and not a Gly). Recent work revealed the addition of one cytosine base at position 4,227 in the MMO Y gene sequence, affording the appropriate frameshift (D. E. Coufal and S.J.L.). The derived amino-acid sequence was consistent with the electron density map, and includes two more amino acids at the C terminus (Trp 363-Ile-Glu-Asp-Tyr-Ala-Ser-Arg-Ile-Asp-Phe-Lys-Ala-Asp-Arg-Asp-Gln-Ile-Val-Lys-Ala-Val-Leu-Ala-Gly-Leu-Lys 389). In addition, 900 water molecules have been included in the refinement. The current R-value is 0.170 to 2.2 Å resolution, with root-mean-square deviations from ideal bond distances and angles of 0.013 Å and 2.9°, respectively.

^{*} $R_{\text{merge}} = \sum |I_{\text{obs}} - I_{\text{avg}}| / \sum I_{\text{obs}}$, where the summation is over all reflections.

[†] Phasing power is given by r.m.s. ($|F_H|_{\text{calc}}|$)/r.m.s. (ϵ), where r.m.s. ($|F_H|_{\text{calc}}|$) is the root-mean-square calculated heavy-atom structure factor and r.m.s. (ϵ) is the root-mean-square lack of closure error calculated by the program PHASE (CCP4 program package, SERC, Daresbury, UK).

[‡] PIP, di- μ -iodobis(ethylenediamine)diplatinum(II) nitrate; TAMM, tetrakis(acetoxymethylmercuri)methane; EMTS, sodium ethylmercurithiosalicylate.

phenoxide³¹ ions to the dinuclear metal centre has been reported.

Each iron atom is also coordinated to a histidine δ N atom, Fe1 to His 147 and Fe2 to His 246, and Fe1 is ligated to a monodentate carboxylate, Glu 114, a semi-bridging (see later) carboxylate, Glu 144, and a water molecule. A terminal H_2O or OH^- ligand was previously identified in the proton ENDOR spectrum of the mixed-valent hydroxylase²⁰. The coordination sphere of Fe2 is similarly occupied by two monodentate carboxylates, Glu 209 and Glu 243, the uncoordinated oxygen atom of the latter being hydrogen-bonded to the water ligand on Fe1 (Fig. 4). Interestingly, Glu 209 is replaced by an aspartic acid in the *M. trichosporium* hydroxylase, a substitution that may help to explain subtle differences in the reactivities of the two enzymes^{32,33}. Taking the terminal monodentate ligand as water and the bridging ligand as hydroxide ion renders the overall active site charge neutral.

The present Fe2–O distances for Glu 144 are 2.7 Å and 2.6 Å in the two subunits, respectively, values which approach coordinating distances and which lead us to characterize this ligand as semi-bridging. Perhaps in the absence of acetate, Glu 144 would be a normal bridging carboxylate. Moreover, a glutamine (Gln 140) is hydrogen-bonded to both Glu 144 and Glu 209, which could weaken the bond between Glu 144 and Fe2. The semi-bridging mode of Glu 144 in our structure, characterized by a normal Fe1–O bond length and a much larger distance for Fe2–O, is a manifestation of the multiple coordination modes of carboxylate ligands to dinuclear metal centres, a phenomenon referred to as the carboxylate shift³⁴. The overall coordination

environment of the dinuclear iron centre is consistent not only with the EXAFS and ENDOR results, as already mentioned, but also with previous optical, electron paramagnetic resonance (EPR) and Mössbauer spectroscopic studies⁶. The present structure also accounts for the spectroscopic properties of the *M. trichosporium* hydroxylase, including the asymmetry of the di-iron centre²¹. It is likely that the two active sites are quite similar.

Figure 5 shows features of the second coordination sphere of the dinuclear iron centre. The ϵ -N atoms of both histidine ligands are hydrogen-bonded to negatively charged aspartic acid residues as anticipated²⁴. These interactions will raise the pK_a value of the bridging hydroxide ligand in the hydroxylase compared to the R2 protein, which has only one such hydrogen-bonded aspartic acid residue²⁶. This difference, together with the bound exogenous acetate anion, helps explain why the oxidized hydroxylase contains a hydroxo bridge rather than the oxo bridge found in the R2 protein.

Access to and composition of the active site

MMO hydroxylase catalyses the oxidation of many hydrocarbon substrates, including small molecules like methane as well as more bulky compounds such as 2,2-diphenylmethyl-cyclopropane³³. The ability to bind and retain a substrate as small and nonpolar as methane is one of the more intriguing properties of this enzyme. As dioxygen is activated for incorporation into substrate at the di-iron centre, the substrate binding site must occur close to this dimetallic core.

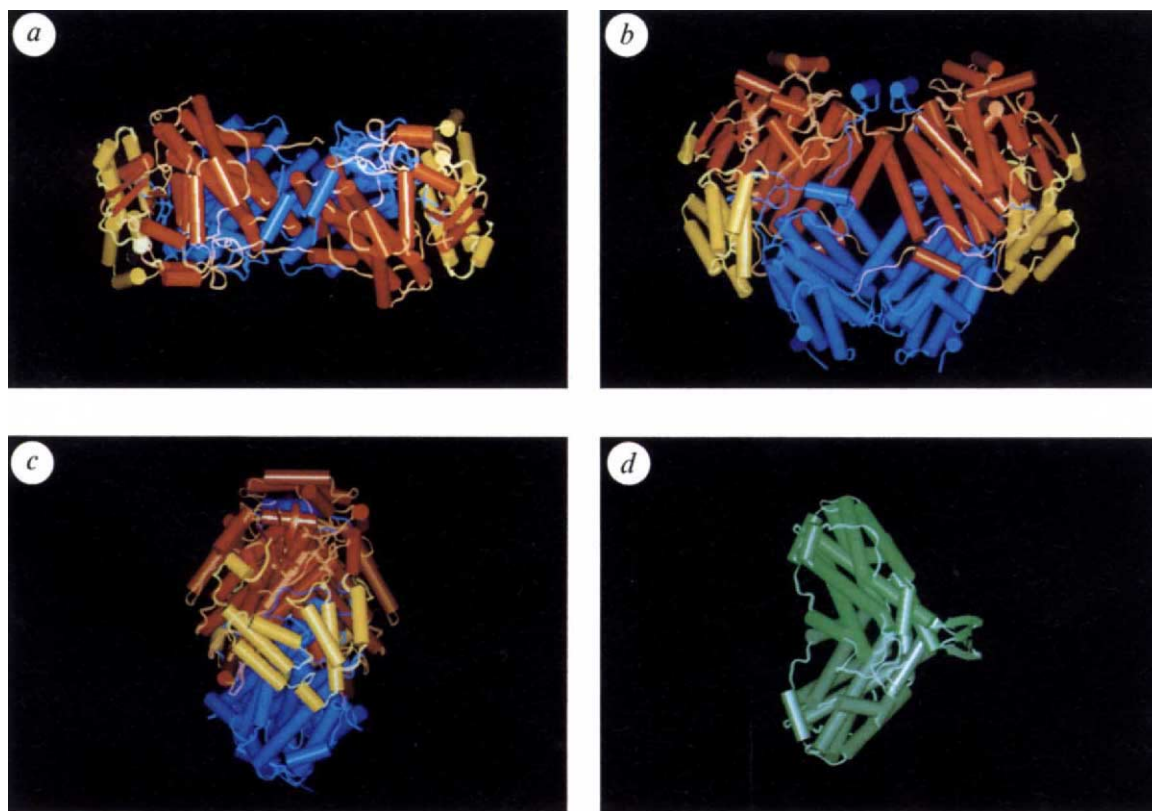


FIG. 1 Structure of the hydroxylase (O^{54}). *a*, Viewed down the molecular twofold symmetry axis. The α -subunits are shown in red, the β -subunits in blue and the γ -subunits in yellow. *b*, Viewed with the twofold axis running vertically. The two α -subunits barely contact one another, whereas contacts between the two β -subunits are extensive. There is a wide canyon formed between the two $\alpha\beta$ halves of the dimer, with an open space in the centre. The α - and β -subunit folds are very similar, despite the lack of sequence homology. The $\alpha\beta$ protomer is formed by interactions between helices which result in a shape that resembles a

heart. The γ -subunit sits at the tip of this heart. The $\alpha_2\beta_2\gamma_2$ dimer thus results from two hearts coming together to form a larger heart. *c*, Alternative view with the twofold axis running vertically. The six longer helices of the γ -subunit can be seen to lie approximately across the $\alpha\beta$ interface in this view. *d*, The ribonucleotide reductase R2 dimer viewed with the twofold axis running horizontally. The shape of this molecule can readily be seen to resemble the two halves of the hydroxylase.

As indicated in Fig. 5, this investigation reveals a cavity at the iron centre. The two iron atoms and the coordinating ligands are located on one internal surface of this cavity, the rest of which is, with the exception of Thr 213, filled with hydrophobic side chains (residues Ile 217, Ile 239, Phe 236, Leu 110, Phe 188, Ala 117, Phe 192, Leu 204, Gly 208). There is also a hydrophobic cavity in the R2 protein at the iron centre²⁶, but that cavity is smaller than the one found in the hydroxylase. This difference is consistent with the fact that the R2 protein does not bind exogenous substrates, but instead catalyses the oxidation of Tyr 122, located in the pocket, to a catalytically essential tyrosyl

radical. The residue in the hydroxylase active site corresponding to Tyr 122 in R2 is Cys 151. This cysteine resides in the back of the cavity and is accessible in the pocket because it binds a mercury atom in the ethylmercury derivative.

Although there is no direct path to the active site from the surface of the protein, significant hydrophobic pockets occur in the α -subunit between the catalytic centre and the second domain which may provide a route for substrate to access the dinuclear iron centre. The largest of these pockets is adjacent to the active-site cavity and could become directly linked by slight conformational changes in either Phe 188 or Thr 213 side chains.

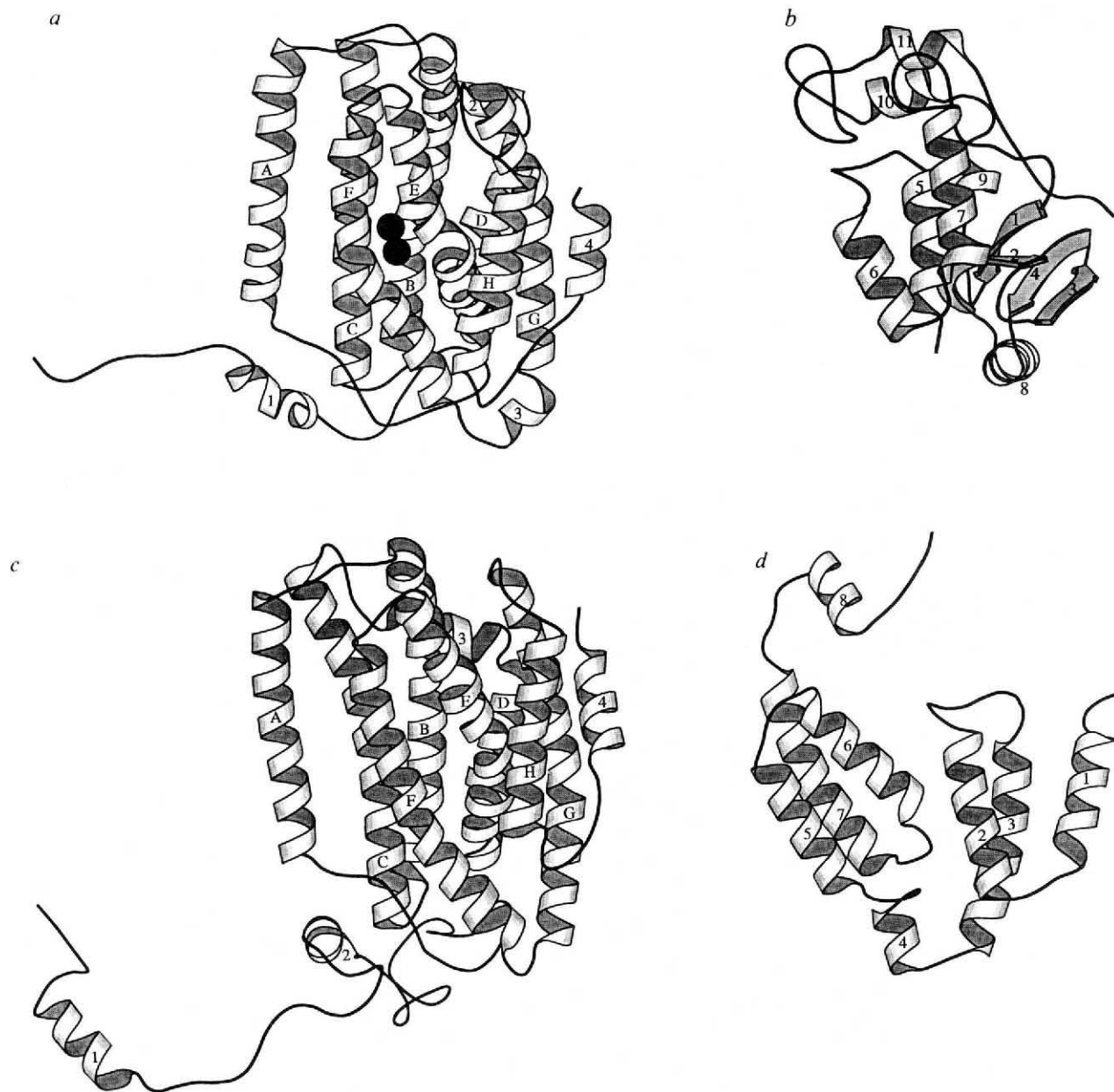


FIG. 2 Secondary structure of the hydroxylase (Molscript⁵⁷). *a*, The first domain of the α -subunit, which is all helical. The principal helices are labelled A–H in accord with the nomenclature used for the R2 protein. The iron-coordinating ligands are derived from helices B, C, E and F. The principal helices extend through the following residues: α A 63–89; α B 97–127; α C 131–161; α D 180–193; α E 197–226; α F 231–257; α G 267–294; α H 301–321. *b*, The second domain of the α -subunit, which begins at helix 5. The two β hairpin structures lie close to the

surface. *c*, The β -subunit, in which the helices in the bundle are again labelled A–H, to emphasize the similarity to the α -subunit. The principal helices extend between the following residues: α A 101–125; α B 132–164; α C 167–199; α D 219–233; α E 236–267; α F 271–301; α G 305–337; α H 344–363. *d*, The γ -subunit. The arrangement of the first four helices resembles that of the last four, resulting in pseudo twofold symmetry (Fig. 1d).

The shortest pathway from the active site to the surface of the protein lies between helices E and F, affording an alternative route to the dinuclear iron centre. Both pathways would depend on dynamic events such as protein breathing or conformational changes which could be induced by the binding of coupling protein and the reductase.

Catalytic mechanism

Although our structure determination does not directly provide information about the catalytic mechanism it does offer some interesting clues. Some of these observations, and those described below, were made previously from a model based on sequence homology to the R2 protein²⁴. The occurrence of Cys 151 in the space occupied by the tyrosyl radical in the R2 protein suggests a redox role for the sulphhydryl group during catalysis, one possibility for which is detailed elsewhere (A. L. Feig and S.J.L., manuscript submitted). The coordination positions of the terminal water and bridging acetate ligands delineate likely binding sites of dioxygen to the reduced hydroxylase, of peroxo and oxo ligands in putative intermediates, and of methoxide ion as well as the oxidation products of other substrates. As indicated in Fig. 5, this region of the active site contains several hydrophobic amino-acid side chains. Perhaps the methane molecule, or the methyl group of another substrate, is clamped into position by these residues near the activated, oxygenated di-iron centre during turnover. The adjacent Thr 213, the only protonated side chain other than cysteine in the pocket, could supply protons needed during catalysis in much the same manner that has been proposed for Thr 252 in the analogous haem enzyme cytochrome P450_{CAM} (refs 35, 36).

Comparison to cytochromes P450

Cytochromes P450, to which the MMO hydroxylase has often been compared, also activate dioxygen for incorporation into a wide variety of substrates. In P450_{CAM} there is a hydrophobic substrate-binding pocket close to the oxygen activation site on the haem^{35,36}. The sequences of the different cytochromes P450 are quite divergent, and there are just two conserved residues found in the substrate-binding pocket, Gly 249 and Thr 252 (ref. 37). These residues are located on the very long I helix at a position where there is an unusual distortion due to an extra residue in the helical turn (a π -turn). When Thr 252 is changed to an alanine, the mutant P450 is far less efficient at oxidation. The X-ray structure of this mutant protein reveals further distortion of the helix and greater access of solvent molecules to the

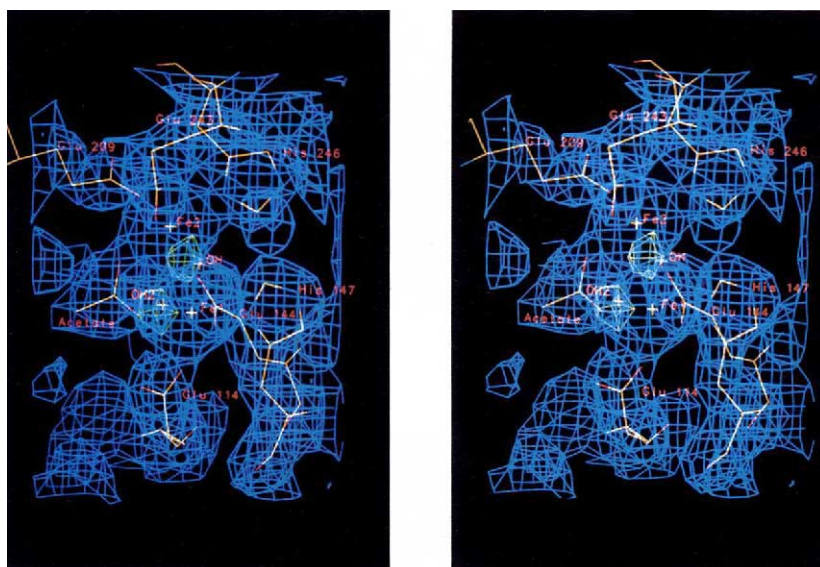
active site. This feature may account for the uncoupling of the reaction, leading to the production of hydrogen peroxide and water instead of hydroxylated product³⁶. Thr 213 in the active site cavity of the MMO hydroxylase is located at a π -turn in helix E, like that in cytochrome P450. This feature may well be an archetypal structural motif in dioxygen activation centres, the function of which may either be structural, stabilizing the helical distortion through intrahelical hydrogenbonding, and/or catalytic, controlling the delivery of protons³⁶.

Component interactions

As the coupling protein changes the redox potential of the di-iron centre and greatly perturbs the EPR spectrum of the mixed valent hydroxylase^{38,39}, its binding domain is expected to lie proximal to the active site. In the absence of coupling protein and reductase, substrate oxidation can occur if hydrogen peroxide is added to the diferric hydroxylase or if dioxygen is added to the diferrous hydroxylase (single turnover)^{40,41}. Binding of the coupling protein, however, dramatically affects both the rate and regioselectivity of substrate oxidation⁴⁰⁻⁴². For example, the addition of coupling protein induces at least a 30-fold increase in the rate constant for the formation of 4-nitrophenol from the substrate nitrobenzene, as compared to a single turnover reaction with nitrobenzene⁴¹. These experiments strongly suggest that the coupling protein causes a conformational change in the substrate-binding site. A molecular mechanism that could account for the effects of the coupling protein is a perturbation in one or more of the helices containing ligands that coordinate to the di-iron centre. Such a conformational change could alter the shape of the hydrophobic cavity and effect significant changes in iron coordination, most probably in the form of carboxylate shifts³⁴. By contrast, the binding of reductase neither changes the spectroscopic properties of the iron site nor affects product distributions, but has been shown by fluorescence studies to interact with both the hydroxylase and the coupling protein³⁹.

The structure of the hydroxylase reveals a possible binding site for the coupling protein and the reductase, namely the extended canyon formed between the $\alpha\beta$ pairs of the dimer. The iron centre is located behind helices E and F, which are exposed in this canyon. The coupling protein could modulate the coordination of the iron centre through interactions with these helices. The iron ligands most likely to be affected are Glu 209 and Glu 243. As two moles of coupling protein bind to each $\alpha_2\beta_2\gamma_2$ dimer for optimal activity³⁹, one molecule of coupling protein

FIG. 3 Final $2F_o - F_c$ electron density map in the vicinity of the di-iron centre at 2.2 Å resolution shown in blue (O^{54}). Superimposed in green is the $F_o - F_c$ difference map showing the hydroxo bridge and water molecule coordinated to Fe1. For the latter map, F_c was calculated from a model refined without including the hydroxo bridge and water molecule. The green contours are 3 times the standard deviation of the map.



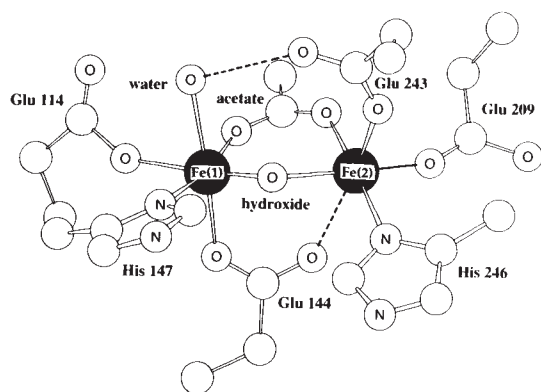


FIG. 4 Schematic representation of the dinuclear iron centre.

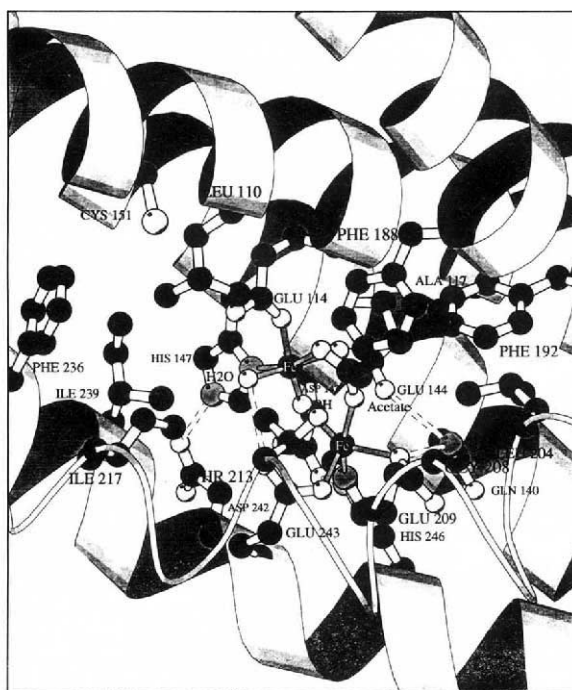
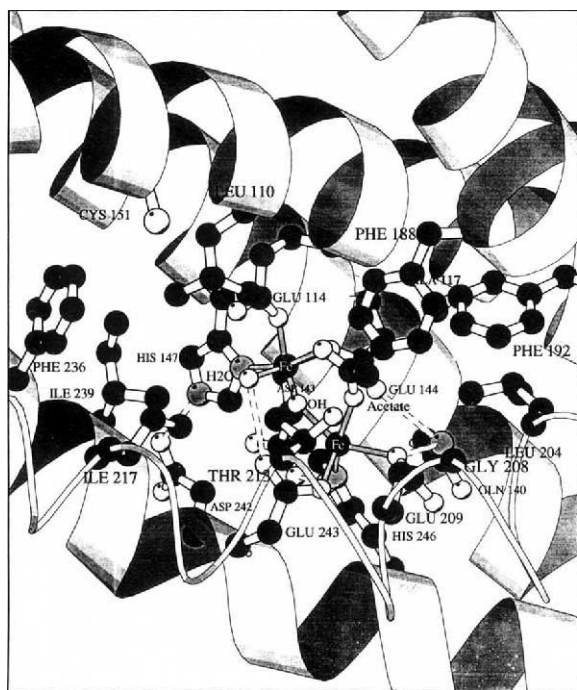


FIG. 5 Stereo view of the active site (Molscript⁵⁷). The di-iron centre is located in a cavity composed of hydrophobic residues (Ile 217, Ile 239, Phe 236, Leu 110, Phe 188, Ala 117, Phe 192, Leu 204, Gly 208). Each

coordinated histidine is hydrogen-bonded to an aspartic acid, His 147 to Asp 242 and His 246 to Asp 143. There is a cysteine, Cys 151, in the back of the cavity, in the same position as Tyr 122 in the R2 protein.

would bind in each of the two canyons related by the non-crystallographic twofold symmetry axis.

Relevance to other non-haem di-iron proteins

Alignment of the *M. capsulatus* and *M. trichosporium* hydroxylase α -subunit sequences with that of the R2 subunit of ribonucleotide reductase²⁴ correctly predicted several details of the iron coordination, despite the fact that the overall homology is very weak. Several other non-haem iron proteins involved in reactions using dioxygen share some sequence homology with the hydroxylase and with the R2 protein. Phenol hydroxylase⁴³ and toluene-4-monooxygenase⁴⁴ show extensive homologies to the hydroxylase, and stearyl-ACP desaturase¹⁰ and ruberythrin⁹ each contain two Glu-X-X-His sequences, analogous to the sequence containing the iron ligands in the hydroxylase and in the R2 protein. These proteins all use dioxygen for oxidation reactions with the exception of ruberythrin.

In conclusion, we now have the detailed molecular structure of a key component in the remarkable machine assembled by nature to hydroxylate methane, the most chemically inert hydrocarbon. The hydrophobic pocket at the active-site cavity indicates how methane might bind to the catalytic centre in the

hydroxylase, and identification of residues in the first and second coordination spheres of the two iron atoms will facilitate interpretation of ongoing kinetics and time-resolved spectroscopy of the catalytic mechanism. Finally, the structure provides insight into where the other two components of this machine, the reductase and coupling protein, might interact to transfer electrons and regulate the rate and regioselectivity of the hydroxylation chemistry. □

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LETTERS TO NATURE

Large-scale solar-wind structure near the Sun detected by Doppler scintillation

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STUDIES of the solar wind in the inner heliosphere (between the solar-wind source surface at ~ 0.01 AU and 0.3 AU) are hampered by the lack of *in situ* spacecraft measurements. Radio propagation measurements—using both natural^{1–7} and spacecraft^{8–14} radio signals—have provided many insights, but information on large-scale solar-wind structure inside 0.3 AU that can be related to coronal features or direct spacecraft measurements at larger distances has nevertheless remained elusive. Here we report the detection of solar-wind structure between 0.08 and 0.53 AU, based on the response of the 13-cm radio signals from the Pioneer Venus Orbiter to electron-density fluctuations and solar-wind speed within this region. These Doppler scintillation measurements were made during the late declining phase of the most recent cycle of solar activity, when the solar wind exhibited recurrent high-speed streams. Near 0.5 AU, we find narrow regions of enhanced scintillation which appear to be associated with compressed plasma at the leading edges of these streams, consistent with previous scintillation measurements^{15,16}. Inside 0.2 AU, however, scintillation enhancements are conspicuously absent from the fast streams, and instead occur in regions where the average solar wind is slow. They exhibit high variability, and appear to be the interplanetary manifestation of coronal mass ejections. The plasma structures giving rise to these enhanced scintillations apparently undergo significant evolution inside 0.3 AU.

Unlike natural radio sources, spacecraft radio signals are coherent, so that phase or Doppler scintillation can be observed

as well as intensity scintillation^{17,18}. Doppler scintillation responds to electron-density fluctuation and solar-wind speed transverse to the radio path. While Doppler scintillation is an integrated line-of-sight measurement, the approximately R^{-2} fall-off in electron-density fluctuation with heliocentric distance R (ref. 17) means that most of the contribution comes from solar wind near the closest approach of the radio path to the Sun. Figure 1 shows the time series of the Pioneer Venus Orbiter (PVO) 13-cm r.m.s. Doppler scintillation σ_D . These Doppler

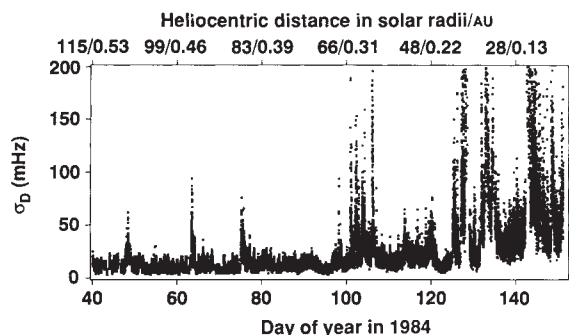


FIG. 1 Time series of 3-min r.m.s. Doppler scintillation σ_D (refs 17, 18). The 2,209 hr of data (82% duty cycle) cover nearly 4 months before the 15 June 1984 (day 167 in 1984) superior conjunction, and encompass Carrington rotations (a coordinate system that rotates with the Sun) 1745–1748, when the PVO radio path probed 18–115 solar radii or 0.08–0.53 AU (corresponding heliocentric distances are indicated top of figure) near the ecliptic plane and off the west limb of the Sun. Several distinct features are apparent in these Doppler measurements. First, solar-wind structure is more pronounced inside 0.3 AU, a region so far excluded from direct spacecraft measurements. Second, alternating regions of quiet and enhanced scintillation, separated by sharp boundaries, are clearly evident. Third, the fine structure in the enhanced regions exhibits abrupt variations, indicating the presence of high velocity shears and/or steep density fluctuation gradients. Fourth, the conspicuously quiet scintillation regions are depressed and marked by pronounced local minima.

scintillation measurements bridge the observational gap between optical measurements of the solar corona and measurements in solar wind. Although *in situ* plasma measurements from spacecraft that are radially aligned with the region probed by scintillation measurements are best suited for correlation studies¹⁹, none were available for this interval, so we compared our observations with synoptic maps of solar-wind parameters, based on six-month averages of solar-wind observations by the Interplanetary Monitoring Platform (IMP)-8 and PVO spacecraft. These maps provide an explicit picture of the average solar-wind stream structure between the heliographic latitudes of $\pm 8^\circ$ during solar minimum conditions²⁰.

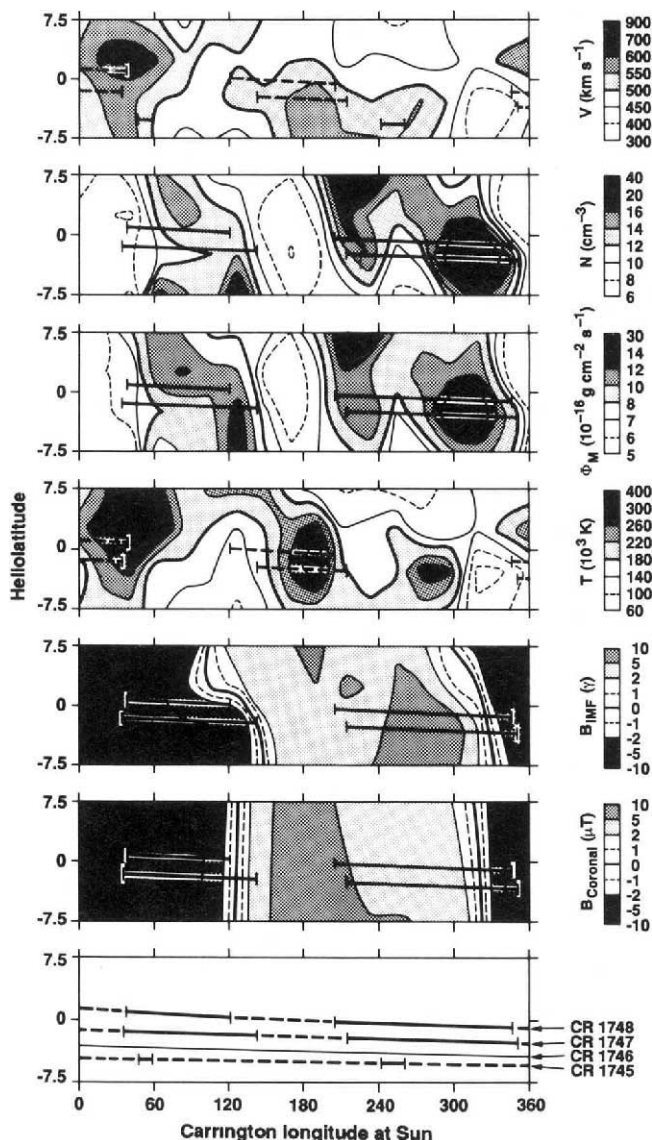


FIG. 2 Synoptic maps of average solar wind and coronal parameters for 6 months beginning on 5 March 1984 (day 65 in 1984) (ref. 20). The solar-wind speed V , density N , mass flux Φ_M , temperature T , the component of interplanetary magnetic field along the Parker spiral B_{IMF} , and the radial component of the coronal field $B_{coronal}$ (J. T. Hoeksema, personal communication) are plotted against heliographic latitude and longitude (Carrington coordinates). Bottom panel shows tracks of the Doppler scintillation observation point for Carrington rotations 1745–1748. Solid and dashed lines, shown on the observation tracks for Carrington rotations 1745, 1747–1748 and superimposed on relevant synoptic maps, correspond to the periods of disturbed and quiet scintillation observed in Fig. 1, respectively, and which are defined in Fig. 3.

Figure 2 shows synoptic maps of average solar-wind and coronal parameters for six months beginning 5 March 1984. Alternating large-scale (and long-lived) regions of fast ($\geq 450 \text{ km s}^{-1}$) and slow ($\leq 450 \text{ km s}^{-1}$) solar wind are clearly evident. Solid and dashed lines corresponding to periods of disturbed and quiet scintillation in Fig. 1, respectively, are superimposed on these maps. Near the Sun, within 0.3 AU (Carrington rotations 1748 and 1747), these maps show that: (1) the quiet scintillation regions span narrower ranges of longitude ($40\text{--}70^\circ$) than the disturbed regions ($90\text{--}150^\circ$), (2) the quiet and disturbed periods generally coincide with low-density high-speed streams (with low mass flux and high temperature) and high-density slow solar wind (with high mass flux and low temperature), respectively, and (3) the disturbed regions lie near but tend to be east (owing to compression) of both the coronal neutral line (the boundary between positive and negative coronal field polarities) and the heliospheric current sheet (the boundary between positive and negative interplanetary magnetic field polarities). It is worth noting that plasma measurements by the interplanetary probe Helios near 0.3 AU also show density fluctuations in the average slow solar wind that are larger than those inside the fast streams, and are significantly diminished by 1 AU (ref. 21).

Preliminary comparisons with simultaneous observations conducted by the Solwind coronagraph on the P78-1 satellite indicate that the scintillation enhancements within 0.3 AU are closely associated with coronal mass ejections (CMEs) observed off the west limb (N. R. Sheeley Jr, personal communication), which is consistent with previous individual and general comparisons between CMEs and scintillation transients^{18,22,23}. Near the Sun, where the Parker spiral angles are small and significant evolution has not taken place, the scintillation enhancements appear to be due to disturbances or transients associated with CMEs and convected outwards by the solar wind. These might be related to the high levels of variability in properties such as helium abundance and proton temperature^{24,25}, and compressive magnetohydrodynamic fluctuations (resembling those of fully developed turbulence^{21,26,27}) revealed by *in situ* observations of the slow solar wind beyond 0.3 AU. The observation that enhanced scintillation near the Sun occurs only in regions where the average solar wind is slow, near the coronal neutral line and heliospheric current sheet, and appears to be associated with the interplanetary manifestation of CMEs, strengthens the case for a close connection between mass ejections and large-scale closed magnetic structures in the corona^{28,29}.

Because σ_D is approximately proportional to $R^{-1.5}$ (ref. 17), corresponding to density fluctuations that vary as R^{-2} , we have scaled the values of σ_D to 1 AU by multiplying by $R^{1.5}$, and have mapped the results to the Sun by assuming a constant radial solar-wind speed of 450 km s^{-1} . The resulting scaled values of σ_D are shown in Fig. 3 for Carrington rotations 1745–1748. A gradual erosion of the fine structure associated with the enhanced scintillations with increasing heliocentric distance is apparent inside 0.3 AU, and is quite pronounced by 0.4 AU (Carrington rotation 1746). This erosion is presumably a consequence of dynamic effects (for example, turbulent decay³⁰ and fading of CME-associated slow-mode structures^{31,32}) as the solar wind and its embedded structure flows outwards from the Sun. However, it could also reflect temporal variations of the wind itself, an ambiguity that comparisons with the Solwind coronagraph measurements will help resolve. Still, the general absence of fine structure beyond 0.3 AU in Doppler scintillation measurements made during the solar minimum period of 1984–1987 argues against temporal variations.

Near 0.5 AU (Carrington rotation 1745) the Doppler scintillation measurements are significantly different from those closer to the Sun, but similar to that seen by metre-wavelength intensity scintillation measurements near 0.5 AU (refs 15, 16). The broad regions of enhanced but variable Doppler scintillation have all but disappeared, and narrow regions of enhanced scintillation now predominate. These narrow regions, such as those during

Carrington rotation 1745 that are superimposed on the synoptic map of solar wind speed in Fig. 2, coincide with the leading edges of the high-speed streams, and are therefore associated with compressed plasma, as in the case of intensity scintillation enhancements^{15,16}. Because solar-wind structure may be expected to undergo substantial evolution by the time it reaches 0.5 AU, attempts to associate scintillation transients^{33,34} or density enhancements³⁵ observed beyond 0.5 AU with events or features at the Sun can be highly uncertain. Also, because we find that high-speed streams are associated with regions of conspicuously quiet scintillation near the Sun, at least during solar minimum, coronal holes³⁶ (regions of low plasma density and open magnetic field that are the sources of high-speed streams) are probably not the direct source of intensity scintillation disturbances observed beyond 0.5 AU. This contradicts previous studies^{33,34}.

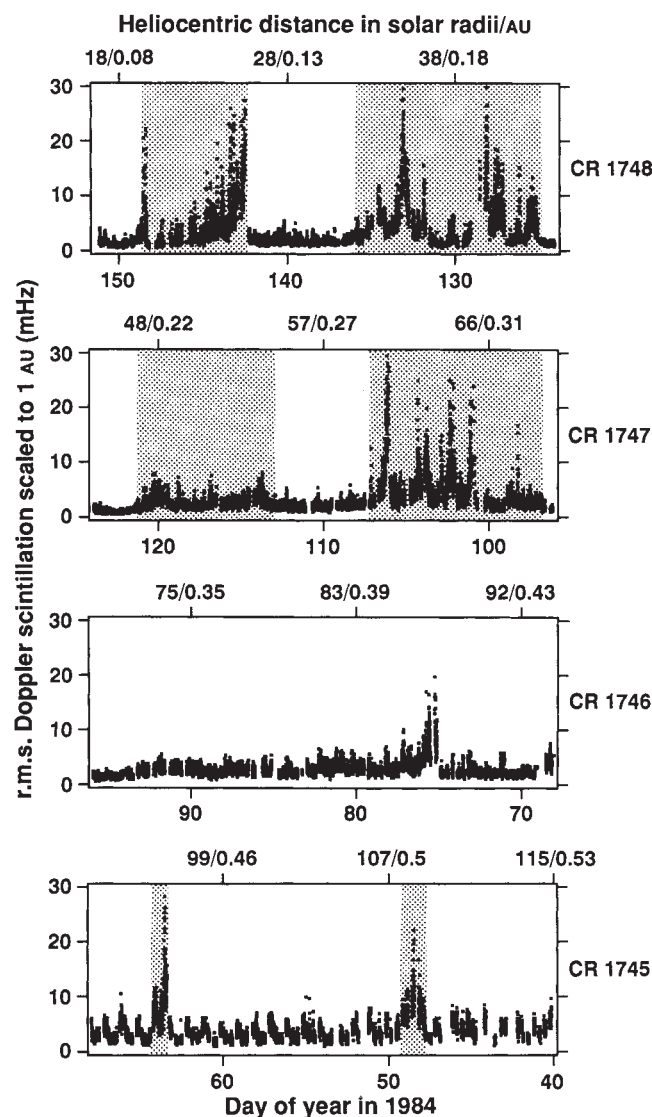


FIG. 3 Time series of normalized σ_D corresponding to Carrington rotations 1745–1748, obtained by multiplying the values of σ_D in Fig. 1 by $R^{1.5}$, and referring them back to the Sun assuming a constant radial solar-wind speed of 450 km s^{-1} . Time axes are reversed and panels displayed in reverse order of Carrington rotation (CR) number so that heliocentric distance increases from left to right. Corresponding heliocentric distances in solar radii and AU are indicated at top of the panels. Shaded regions highlight the periods of disturbed or enhanced scintillation which have been superimposed on the relevant synoptic maps in Fig. 2. Note that the regions closer than 0.3 AU are significantly broader than those near 0.5 AU.

In conclusion, the Doppler scintillation results show strong variations which are strikingly different inside 0.3 AU and outside 0.5 AU. Near the Sun, these variations originate throughout the region where the average solar-wind speed is low, and are probably associated with coronal mass ejections. Further from the Sun, the variations are concentrated at the leading edges of high-speed streams where the plasma is compressed. This evolution of Doppler scintillation from a response dominated by embedded structures near the Sun, to a response dominated by stream interactions further from the Sun, is consistent with current views^{25,36,37} and theoretical models³⁸ of the solar wind.

The ability of Doppler scintillation to identify conspicuously quiet fast wind near the Sun makes it possible to carry out new investigations of this important region. Of particular interest is the use of Doppler scintillation and spectral broadening measurements¹⁰ to study the power spectrum of electron-density fluctuations and its radial evolution, as well as the use of Faraday rotation measurements to study magnetic field fluctuations in a region where alfvénic turbulence is expected to be prevalent^{12,21}. Simultaneous Doppler scintillation measurements may also shed light on the putative regions of enhanced turbulence inferred from scintillation index measurements^{39,40}. Coordination and correlation of Doppler scintillation measurements (by missions such as Galileo⁴¹) with *in situ* particle and field measurements by Ulysses will improve our understanding not only of large-scale solar-wind structure near the Sun, but also of its evolution out of the ecliptic plane. □

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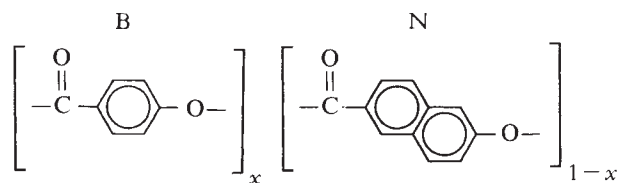
Sequence segregation in molten liquid-crystalline random copolymers

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THE polymerization of two or more types of monomer to form random copolymer molecules yields commercially important thermotropic liquid-crystalline polymeric materials¹. The limited degree of crystallization observed for these random copolymers is thought to occur through a process of segregation: random but similar sequences of the different monomer units on adjacent chains come into register to form a layered structure with crystalline periodicity perpendicular to the chain axes but with aperiodicity parallel to the chains, giving 'non-periodic layer' crystals^{2,3}. If, as has been believed, the melt is a nematic liquid crystal⁴, the question of how the necessary large-scale molecular rearrangements can occur even when crystallization is rapid is puzzling. Here we report the results of a wide-angle X-ray scattering study of a series of oriented molten samples of a class of these materials known as B-N random copolymers, which show that segregated, layered structures are already present in the melt. In other words, the melt contains smectic-type regions within a nematic matrix. The presence of these regions of sequence matching opens up the possibility of controlling the microstructure, and thus the properties, of thermoplastics through the introduction of specifically synthesized non-random sequences into the molecular chains.

B-N copolymers are random copolymers of 1-hydroxy-4-benzoic acid and 2-hydroxy-6-naphthoic acid. The para-linked ester chains are sufficiently straight and rigid to ensure that the melt phase (>285 °C) is liquid-crystalline (LC):



The search for evidence of melt structure in these materials was stimulated by observations that both of the parent homopolymers display phases at high temperatures that resemble smectic liquid crystals⁵⁻⁸. In each case the segregation is considered to be on the scale of the monomer repeat, and does not involve the chain ends. Despite these indications, the random copolymers have hitherto always been considered to be nematic.

In the wide-angle X-ray scattering (WAXS) experiments, the aim has been to look for 00/ reflections from the melt, and if present, to examine their shapes. These reflections provide information both about the periodicity (or otherwise) along the axes of the polymer chains and the degree to which neighbouring chains are in longitudinal register. In the solid phase of the system under examination, they are aperiodic in position, a consequence of the random distribution of monomers of different lengths (6.3 and 8.4 Å) along the polymer chains⁹⁻¹², so that the *l* index must be taken as a layer line number, rather than a fraction of any unit cell dimension. From fibre diffraction experiments⁹ we know that the B-rich copolymers display 002, 004 and 006 reflections, whereas the N-rich materials show 002, 004, 006 and 008 reflections. For example, the 006 reflection of the polymer with composition ratio (B:N) of 3:1 corresponds in position to the 008 reflection of B-N 1:3, and has a *d*-spacing of ~2.1 Å, representing the longest periodicity common to both monomer units. Generally, the 002 reflections are concentrated

as points on the meridian of the fibre pattern, whereas the 004, 006 and 008 reflections are increasingly spread along the layer lines, due to imperfect registration between adjacent chains¹³. The 002 reflection corresponds to correlations on the scale of a monomer length, so an 002 that is concentrated on the meridian is a good indication that the monomer units have organized themselves into layers; the 'aperiodic' position of this reflection confirms that the sequences contain both units. With our major interest here being in the lateral 'propagation' of random sequences, we have focused particular attention on the lateral breadth of the 002 reflections.

Figure 1*a* and *b* shows transmission diffraction photographs of the high molecular weight (36,000) sample with composition ratio 3:1 in the oriented state under shear. The striking feature is that the 002 reflections are equally clear in both the melt (Fig. 1*a*) and the crystallized (Fig. 1*b*) states. The importance of this finding is to be emphasized. The similarity between the 002 reflections of the solid and melt phases suggests that the melt possesses a similarly segregated structure, and that this segregation occurs in regions which would correspond, at least in terms of volume fraction, to the crystals of the solid polymer. The solid lines in Fig. 1*c* and *d* are meridional and azimuthal traces respectively of Fig. 1*a* (the shearing melt); the dashed lines are the equivalent traces from the quenched sample (Fig. 1*b*). The similarity in the azimuthal spreads is very marked, the value of *P*₂ (the coefficient of the second-order spherical harmonic), being 0.55 ± 0.03 in each case. The sloping background of the 002 peak for the melt is due to the tail of the non-crystalline equatorial component. In the case of the solid sample, this sloping background is less marked as some of the equatorial intensity is now in the crystal peaks. The equatorial 'halo' in the solid sample now peaks at a slightly higher angle, and the 002 reflection is actually at a lower angle, indicating that the conformation of the ester linking groups is somewhat more perfect (giving a slightly longer axial repeat), in the crystalline state. It is important to question whether the interchain register apparent in the melt represents a phase in thermodynamic equilibrium, or whether it is an ordered component of the crystal which has not had sufficient time to melt. For this reason, additional diffraction patterns were recorded after 5 and 10 h continuous shearing in the melt. Comparative meridional and azimuthal scans are shown in Fig. 1*e* and *f* respectively. There is little or no evidence of any change in the profile of the 002 peak, and we conclude that the sequence-matched phase, which is a component of the melt, is stable at 315 °C. Similar experiments of a polymer of lower molecular weight (8,600) gave similar results, the only differences being that the azimuthal width of the 002 reflection decreased by 25% (with respect to the high-molecular-weight sample) and the intensity of the reflection on a meridional scan was 50% higher.

Figure 2*a* is a reflection diffractometer scan of a thin sample of the polymer of molecular weight 6,200 and composition ratio 1:3, homeotropically aligned on a substrate of an irrationally cut silicon crystal. Although the alignment here is not as good as that obtained in the shear cell, the method has the advantage that higher-order meridional peaks can be accurately recorded. As before, the intensity of the 002 reflection is unchanged on quenching from the melt, an aspect emphasized in Fig. 2*b* which shows the 002 peak profiles with the sloping background removed. It is intriguing to note that the intensities of the 006 and, especially, the 008 maxima are actually greater in the melt than in the solid phase. The reduction, on crystallization, of longitudinal chain registration on the 2-Å scale (but not the 6-Å scale) may be associated with the fact that the polymer chains are polar, but not segregated on this basis except in small local groups. The same trends were seen in the B-N copolymers of other compositions and molecular weights examined by this method (composition ratio 3:1, mol. wt. 5,000; 1:1, 5,800; 1:3, 6,200). The fact that the 002 peak also has the same form in the molten and solid states in this experiment (in which the polymer

was quiescent) is evidence that the corresponding effect seen in shear-aligned melt (above) is not a consequence of the shear flow.

Figure 3 illustrates the hierarchical levels of order which occur in these polymers in the solid state^{2,3}. The sequence-segregated regions also possess two-dimensional long-range packing order,

and so consist of aperiodically spaced layers normal to the chain axes. These regions, 'non-periodic layer' (n.p.l.) crystallites, are themselves planar and separated by a long period characteristic of the sample and its thermal history. The segregation within the crystallites is possible only because there are considerable unsegregated regions in between with a transition 'fringe' at the

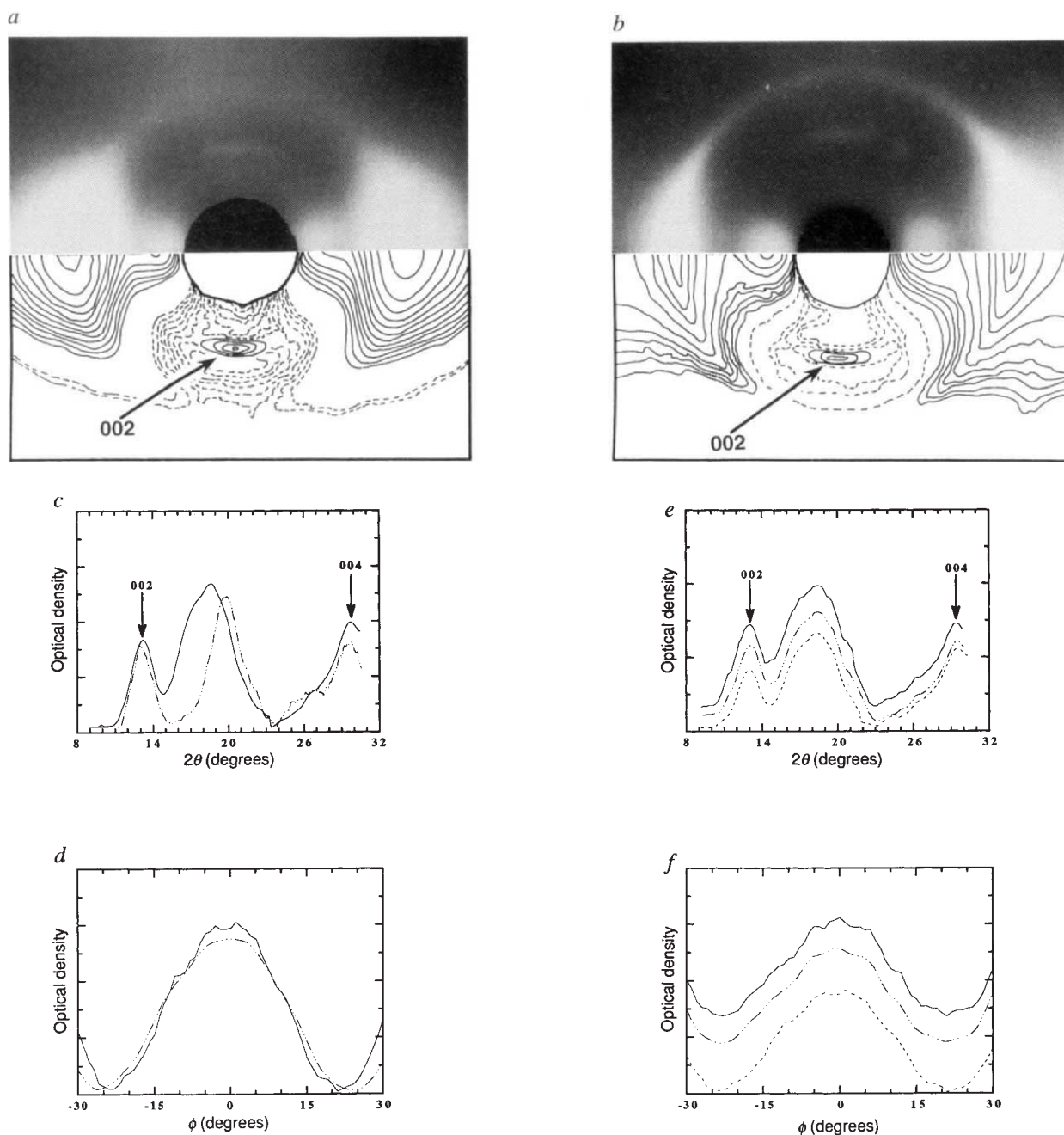


FIG. 1 Transmission X-ray diffraction data of the B-N random copolymer with composition ratio (B:N) 3:1, molecular weight 36,000 and melting point 285 °C, obtained by orienting in the shear cell already described in ref. 24. The shear axis is vertical on the patterns and the shear plane parallel to the photographic film. The samples were prepared by hot pressing at 280 °C for 5 min. All samples were of the same thickness, received the same 15-min X-ray exposure, and the film processing was standardized. Cu K α radiation was used. *a*, Diffraction pattern obtained during shearing at a strain rate of 100 s⁻¹ in the melt phase at 315 °C. The shear-induced orientation is sufficient to reveal the 002 reflection while the diffuse nature of the equatorial scattering is proof that the sample is molten. *b*, Diffraction pattern of the same sample at room temperature (25 °C) after cooling during shearing. Crystalline arcs may now be observed in the equatorial region. The 002 reflection is clearly

visible and has a similar appearance in both photographs. In both cases the lower part of the diffraction pattern has been digitized and contoured. The meridional and equatorial regions are represented by solid contours, and the weaker off-axis scattering is shown as dashed contours. The contours represent larger intensity increments in the equatorial regions than in the meridional. *c*, Meridional (vertical) scans through the molten (full line) and quenched (broken line) patterns. *d*, Azimuthal scans through the 002 reflection for the molten (full line) and quenched (broken line) patterns. *e*, Meridional scans of patterns obtained with exposures commenced after continuous shearing at 315 °C and 100 s⁻¹ for: full line, 0 h; broken line, 5 h; dashed line, 10 h (origins displaced vertically for clarity). *f*, As *e*, but azimuthal scans through the 002 reflection.

boundaries¹⁴. The new diffraction data reported above indicate that the melting of the crystallites involves the loss of the packing order within the planar regions, but not the loss of the associated sequence segregation.

The molten polymer within the segregated regions is, in essence, smectic, while that in between is nematic. But the usual smectic classification is not obviously applicable here because the 00/ peak positions are aperiodic indicating that the layers are randomly disposed (with different thickness corresponding approximately to the different lengths of the monomer units), in marked contrast to the regular and uniform layers of a conventional smectic A phase¹⁵. The layers are also tied together by the polymer chains. Nevertheless it seems that the melt is biphasic, with a combination of smectic and nematic phases.

The observation that sequences are segregated and matched in the melt phase before the process of crystallization removes a significant problem in our understanding of the formation of the n.p.l. crystals. The fact that these crystals form even under the conditions of severest quenching has always seemed at variance with the requirement of the segregation of like sequences into layers on crystallization. The fact that segregation occurs

in the melt avoids this difficulty. Not only is the available time and mobility much greater, but the possibility of transesterification in the melt phase provides an additional means of sequence segregation through the exchange of monomer units between chains. Transesterification involves a chain end 'biting' into a neighbouring molecule to leave a new end, or possibly two adjacent chains simply swapping connections at one point. It is well known and documented in conventional molten polyesters, and has been observed in liquid crystalline systems by a number of authors¹⁶⁻²¹.

The possibility that a chemical mechanism is contributing to segregation has important consequences for the observed size and spacing of crystallites. Electron microscopy has revealed a pronounced regularity of the crystalline long-period in samples oriented in a magnetic field²², (inset micrograph, Fig. 3). A systematic study has shown that the long-period is not related to

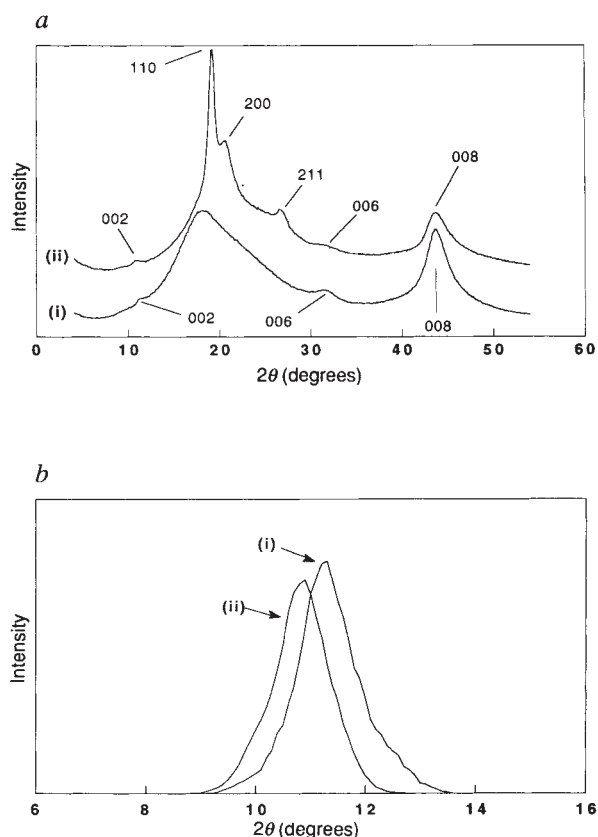


FIG. 2 Reflection diffractometry obtained using a theta-theta diffractometer fitted with a high-temperature stage. The sample with composition ratio (B:N) 1:3, molecular weight 6,200 and melting point 280 °C was prepared by melting onto an irrationally cut single-crystal silicon substrate at ~330 °C and then quenching. Diffraction scans of the melt were made at 305 °C. As with the shear cell experiments, it was necessary to heat rapidly (at 50 °C min⁻¹) to this temperature to prevent the quenched pseudo-hexagonal structure from transforming to a higher melting point orthorhombic phase^{25,26}. Cu K α radiation was used. a, Reflection X-ray diffractometer scans. Trace A, molten sample at 305 °C. The fact that the sample has aligned homeotropically on the substrate means that the reflection scan is broadly equivalent to the meridional sections of Fig. 1a and b. Trace B, The same sample after cooling to room temperature (25 °C). In both cases the prominent meridional reflections are indicated. b, Close-up of 002 reflections from a after background subtraction and smoothing: Trace A, 305 °C; Trace B, 25 °C.

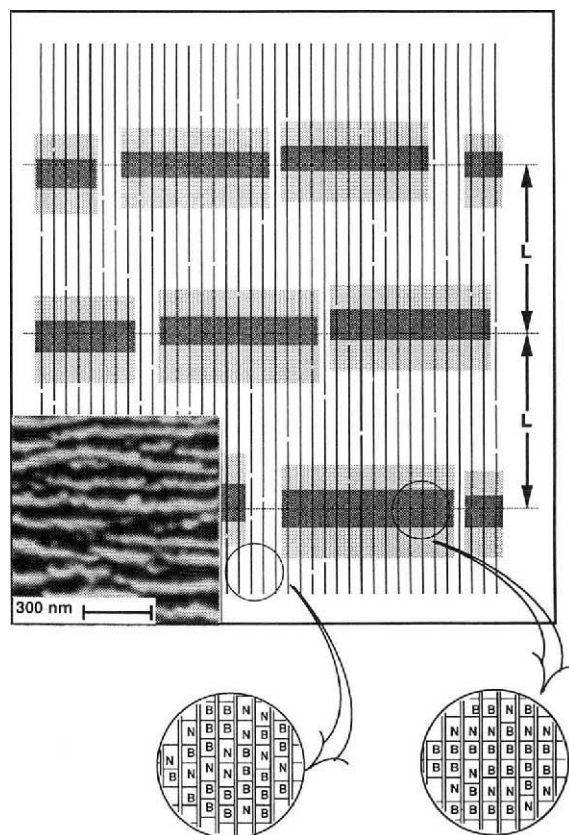


FIG. 3 Schematic representation of the solid phase in the B-N copolymers. The polymer chains, represented by black lines, run vertically. The dark grey regions represent the crystalline core regions, in which the sequences on adjacent chains are thought to match well, whereas the lighter shaded areas indicate the partially ordered fringe region in which the quality of sequence matching gradually decays¹⁴. The expanded circular regions show the chain packing on a monomer scale within the crystalline and non-crystalline regions. The crystal long-period, L , which represents the mean distance between crystal centres parallel to the chain direction, is marked. The only difference between the solid phase, as-drawn, and the melt, is that the periodicity of the spacing between the polymer chains in the crystals will be lost on melting, whereas the segregation on a monomer scale parallel to the chains will be maintained. Inset (not to same scale), scanning electron micrograph of a section from an intermediate-molecular-weight sample of composition ratio (B:N) 3:1 (molecular weight 14,400). The sample was oriented by a 5.67-T magnetic field for 15 min at 300 °C and was treated for 3 min with a permanganate etch²⁷. The regularly spaced crystallites are visible as the light regions. (Micrograph by courtesy of A. Anwer).

the mean length of polymer chains, and recent work²³ has demonstrated that chains of molecular weights of the order of 30,000 will pass through as many as 6 separate crystals along their lengths. If the only mechanism for sorting were diffusion of rigid chains, it would be necessary for sequence matching to occur simultaneously on up to six different sites, with a reduced chance of success. If, on the other hand, transesterification is occurring, then it can be seen that the actual length of a molecule ceases to be of relevance, because the effective molecular weight will correspond to the mean distance between scission points, which will reduce with increasing time in the melt. □

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Electrical conductivity measurements from the GISP2 and GRIP Greenland ice cores

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THE direct-current electrical conductivity of glacial ice depends on its acidity^{1–3}, and can also indicate changes in climate, as ice formed in cold, dusty periods has a high concentration of alkaline dust^{1,4,5}, which significantly reduces the conductivity^{6,7} compared to warmer, less dusty periods. Here we present electrical conductivity records for the Greenland Ice Sheet Project 2 (GISP2) and Greenland Ice-core Project (GRIP) ice cores, drilled 28 km apart to enable direct comparison of the results. The upper parts of both records are consistent with previous evidence from other Greenland cores^{4,8–12} for a stable Greenland climate during the Holocene, and a series of warm events punctuating the last glacial period. However, there is a significant discrepancy between the two records in the bottom 10% of the cores, calling into question recent reports of climate variability in the last interglacial^{4,8} and the penultimate glaciation⁸. At this stage, it is too early to say what exactly is causing the discrepancy, although ice flow may have introduced

some discontinuities into the records. Further work will be necessary to establish how much climatic information it will eventually be possible to extract from the lower parts of the two cores.

The electrical conductivity measurement (ECM) method involves making continuous, high-resolution measurements along the full length of the core. Although in principle other climate proxies, such as oxygen isotope ratio, can be measured in the ice at comparable resolution, this is too intensive to be practicable for the entire core. In addition to confirming the findings from other parameters, the ECM method is therefore useful for identifying regions of the core that should be investigated at high resolution using more labour-intensive parameters. As outlined above, during cold periods glacial ice tends to be dustier, and hence more alkaline than during warm periods. Although this means that the ECM signal is smaller for ice

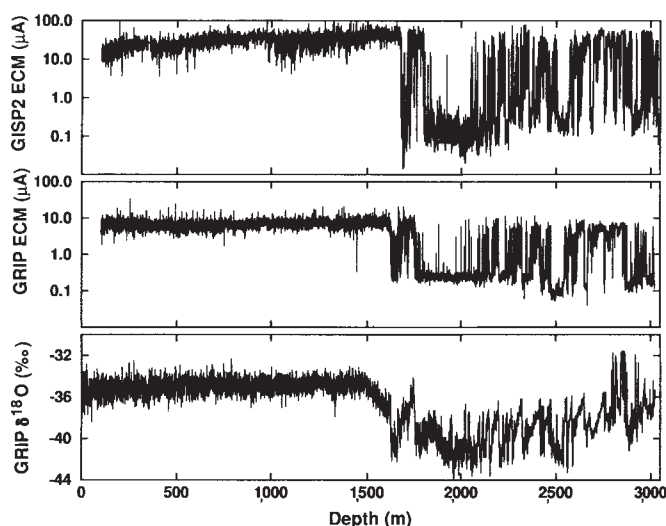


FIG. 1 Climate records from the Summit, Greenland ice cores. The ECM and GRIP $\delta^{18}\text{O}$ plots have resolutions of 10 and 55 cm respectively. The GISP2 ECM signal is the current flowing between two electrodes with a potential difference of 2,100 V; 1,250 V was used with GRIP. This difference does not affect the relative shape of the two ECM records. Volcanic eruptions, which are not visible in this plot because of the scale, will be discussed in a future paper.

deposited during cold periods, we have nevertheless managed to resolve considerable detail in the alkaline ice, including detecting the influence of dust layers that have been observed visually, and which may be annual^{13,14}. Because the ECM response is non-linear and poorly calibrated between systems, the absolute value of the ECM is less significant than the pattern of relative amplitudes.

From the surface to a depth of 2,700 m at GISP2 (2,670 m at GRIP) the electrical conductivity measurement (ECM) records are well correlated, both for large trends that span hundreds of metres (Figs 1 and 2), and abrupt large-amplitude changes that span a few years or less (Fig. 3a and b). The excellent correlation of the ECM records in these parts of the cores give us great confidence in the completeness of the climate record above these depths. However, below the above-mentioned depth, the correlation between the ECM records is reduced (Fig. 4). Many layers <20 m thick do not correlate between the cores, and the interpretation of the record is less straightforward.

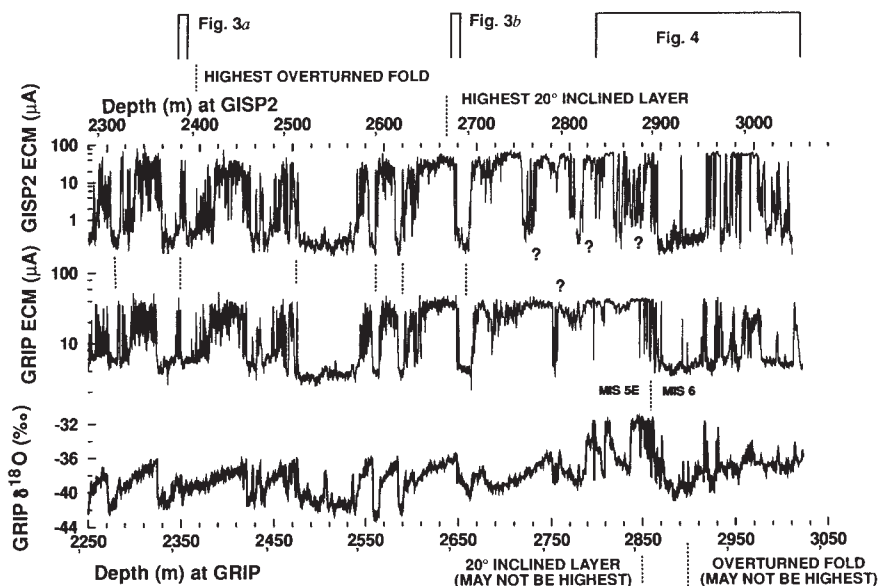
The dust layers that may be annual^{13,14} appear as thin cloudy bands in most of both cores, especially in the sections that accumulated during cold periods. Continuous stratigraphic observations of the GISP2 core reveal that from the surface to 2,200 m depth the bands are undistorted. Below this, small-scale distortions in the record may be indication of larger-scale artefacts associated with ice flow. For example, sinuous wave-like distortions of the bands increase gradually with depth below 2,200 m. The shallowest overturned fold, with a vertical displacement of 1 cm, occurs at 2,400 m. Inclined layers that exceed the detection limit of 8° are first observed at 2,678 m. At GRIP, overturned folds were observed at 2,898 m and inclined layers (4° detection limit) were observed at 2,847 m. These features may also occur higher in the GRIP core, where stratigraphic observations are discontinuous. Folds that contain only a few dust layers may be associated with fold enhancement of annually varying depositional features such as sastrugi (wind induced ridges). In some cases the overturned folds contain many parallel dust layers, indicating that many years' accumulation has been folded and that depositional origins for the features are unlikely. In both cores layer inclinations of >20° were visible intermittently, some sections of which extended for several metres. The inclined layers and small overturned folds raise the possibility that larger folds or other structural features may exist in the lower portions of the ice sheet.

Two general categories of deformation mechanisms are possible; however we should stress that at present we lack any quantitative knowledge of their magnitude at the Summit sites. The

first category results in differential thickness changes^{12,15,16}. Soft layers may have been squeezed into a boudin structure characterized by being thicker, thinner, discontinuous, or a combination of these distortions at different locations. Hiatuses may exist in the stratigraphic record, but the stratigraphy is in chronological order. The second category of deformation mechanisms results in overturned folding¹⁷ or intrusive flow¹⁸, which alters the chronological sequence of the stratigraphy. These mechanisms can duplicate sections of the stratigraphy, so that ice that accumulated at a single time may appear in two or more non-adjointing segments of a core. Both categories of deformation have been extensively studied in geological environments^{19,20} where multiple cores or outcrops make it possible to trace layers for extended distances. We should stress that much less is known about these mechanisms in ice sheets, where observations are more limited²¹, although some observations have been reported^{22,23}. Here we offer some speculation about the possible role that either of these mechanisms may have played at the GISP2 and GRIP drilling sites.

The two drill locations were chosen in different stress regimes to facilitate recognition of anticipated distortions due to ice flow^{24,25}. The GISP2 site is located 28 km west of the present-day ice flow divide²⁶, and has a greater horizontal velocity than GRIP, which is on the present ice divide and dome. It is likely that this has been the situation for a few millions of years, but earlier divide migrations of a few tens of kilometres are likely to have occurred^{24,25,27}. Because of the different locations with respect to the current flow divide, the ice under the sites is in different shear-stress regimes. The different shear-stress regimes will influence the type of deformation that is occurring at the two sites. In the pure-shear regime, associated with the vertical compression and thinning that is now under the ice flow divide at GRIP, boudins can occur. Overturned folds cannot be forming now²⁰, except for centimetre-scale drag features along the upper and lower margins of boudins, or near inclined bedrock. Boudins will alter the thickness of layers, possibly to the extent that some layers are reduced to a negligible thickness¹⁵. The chronological order of the layers will be preserved and features in the ice core are a record (although possibly incomplete) of climate events. In the simple shear regime associated with depth-dependent lateral extension (but no vertical thinning) that is currently occurring in the lower portion of the ice sheet under GISP2, overturned folding can occur²⁰. This folding will alter the chronological order and introduce features into the ice-core record that are not related to climate. Ice that is folded out of chronological sequence may be physically mixed with adjacent ice during the

FIG. 2 Climate records from the bottom of the Summit, Greenland ice cores. The ECM and GRIP $\delta^{18}\text{O}$ plots have a resolution of 10 and 55 cm respectively. The GRIP and GISP2 ECM plots have been averaged over 10 cm. Some stratigraphic tie points between the ECM records are indicated by dashed lines. Features that do not correlate well are indicated by question marks. Low $\delta^{18}\text{O}$ values indicate colder periods. Low ECM values indicate alkaline ice, containing wind-transported carbonate dust. The identification of the marine isotope stage (MIS) 5e/6 contact in the GRIP core is from refs 4 and 8. (The parts of the record shown in expanded scale in Figs 3 and 4 are indicated at the top of the Figure).



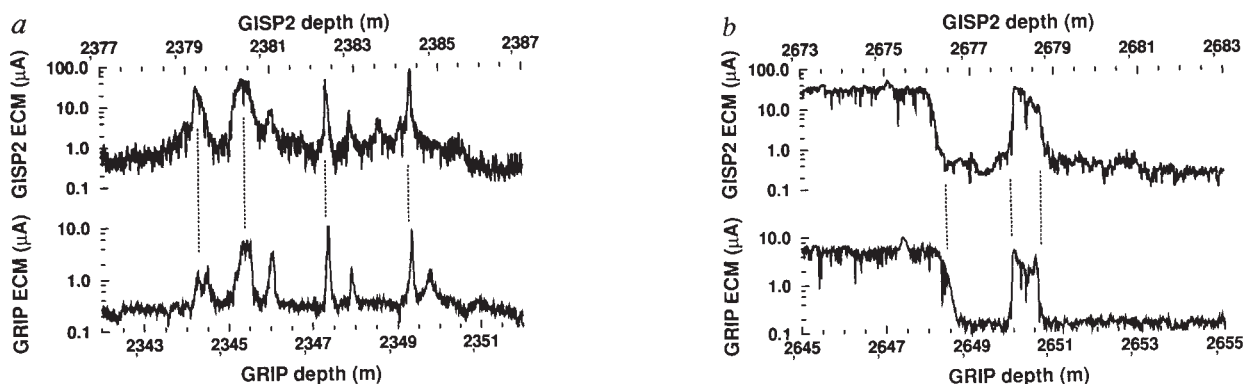


FIG. 3 ECM records of detailed features. The ECM plots have a resolution of 1 cm. Some stratigraphic correlations are indicated by dashed lines. Features thicker than 20 cm correlate between the cores. Poorly understood differences occasionally occur over intervals <20 cm; how-

ever, this occurs much less frequently and is on a scale an order of magnitude or more smaller than the differences that exist in the lowest 10% of the core. Features smaller than 10 cm may be independently influenced by localized variability in deposition and metamorphism.

folding process. If this occurred, the folded ice would no longer be a pure sample of the original unfolded ice.

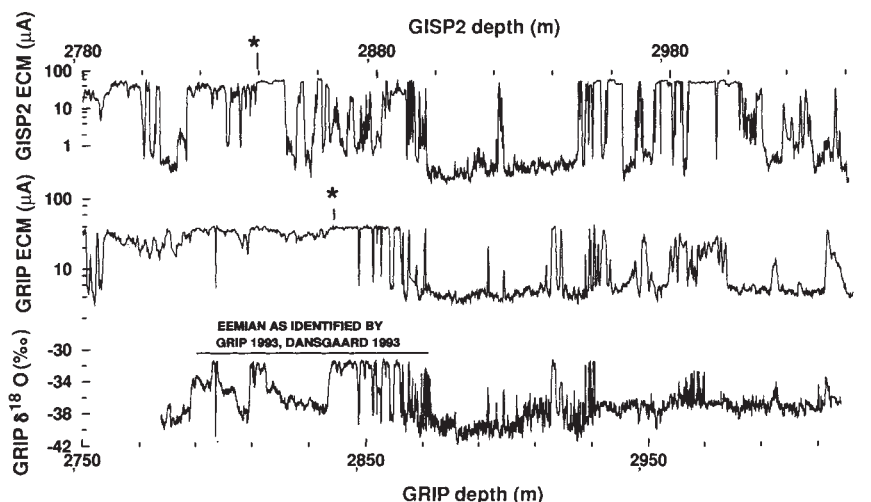
Because the rate of deformation increases with depth, the most recent stress distribution will have had the greatest effect on the magnitude and distribution of deformation in the lowest portions of the ice sheet. Earlier divide migrations of tens of kilometres are likely^{24,25,27}, and would have had a weaker, but not negligible, influence on the deformation of the bottom 10% of the ice. Flow distortions caused by bedrock topography can also initiate overturned folding at off-divide sites regardless of the shear-stress regime. This begins at a distance above the bed that is comparable to the vertical relief of the bedrock topography²⁸, which is 200 m in the Summit region^{29,30}. Folding can also be the result of migration of flow paths associated with changes in ice-sheet geometry¹⁷.

These concepts of deformation mechanisms help us to interpret the stratigraphic differences between the two cores. It is difficult to correlate the 20-m-thick low-ECM layers (occurring in the GISP core around 2,755 m and 2,810 m) with corresponding features in the GRIP core. This could result from the formation of boudins at the GRIP site, which has greatly thinned these low-ECM layers. The formation of boudins is favoured in a pure-shear regime such as GRIP, where the thickness of layers is altered, as compared to the nearly simple-shear regime in the lower portion of the GISP2 core where layer thickness is largely

preserved. In the GISP2 core, and to a lesser degree in the GRIP core, there are numerous thin low-ECM layers that occur just above the clean-dusty ice contact at 2,890 m at GISP2 (2,870 m and the marine isotope stage 5e/6 boundary^{4,8} at GRIP). This is a likely place for the formation of folds, because of the differences in flow properties of ice with different impurity levels^{15,31-34} and the high shear stress that occurs near the bottom²⁶. It is possible that the low-ECM layers seen above this contact are artifacts of overturned folds which have moved dusty ice (low ECM) up and into clean ice (high ECM). Overturned folding occurs predominantly in a simple-shear environment and hence we expect it to be more abundant at GISP2. We note that more of these possible overturned folds of low-ECM ice are observed at GISP2 than at GRIP. Overturned folding may have occurred at GRIP under a previous flow regime associated with a different location of the ice divide^{24,25,27}, producing some of the variability in the GRIP record immediately above the contact at 2,870 m (GRIP depth).

We are confident of the climatic significance of long trends, including the sequence of interstadial events and the associated abrupt transitions, that are preserved above 2,700 m at GISP2 (2,670 m at GRIP). Below this, we speculate that the long low trend from 2,895 to 2,945 m at GISP2 correlates with 2,870–2,914 m at GRIP (marine isotope stage 6 (refs 4, 8)). With the data that are currently available below 2,700 m at GISP2

FIG. 4 Climate records from the bottom of the Summit, Greenland Ice cores. The ECM and GRIP $\delta^{18}\text{O}$ plots have a resolution of 10 and 55 cm respectively. There are many events <5 m thick that do not correlate between the cores. At these depths 5 m of ice contains the accumulation from several millions of years. Stars indicate where the character of the ECM signal changes. Above these transitions, the ECM records show a moderate level of variability when the ECM value is >10 μA . Below these transitions, the ECM records are remarkably uniform when the ECM value is >10 μA . The reduction in the variability of the ECM signal may be associated with centimetre-scale physical mixing during folding, or periods of exceptionally stable climate. The time period identified in refs 4 and 8 as the Eemian interglacial (marine isotope substage 5e) is shown for comparison.



(2,670 m at GRIP), it is not possible to determine the duration of anomalous features thinner than 20 m. In both cores, some of the features near the marine isotope stage 5e/6 boundary^{4,8} that are thinner than 5 m may contain ice that is out of chronological sequence. These findings raise concerns about the interpretation of previously reported climate variations during the Eemian period (marine isotope sub-stage 5e, 2,790–2,870 m at GRIP)^{4,8}. At present we are unable to say which, if either, core contains a more complete climate record.

Detailed consideration of the records may be able to determine the extent of deformation. For example, the several-century age difference (a few tenths of a metre at these depths) between the ice and the gas it contains^{35,36} will result in abruptly changing chemical and gas features being preserved in ice that has different flow properties. Deviations from the anticipated offset between the records of ice chemistry and rapidly responding gases such as methane, may permit identification of features in the records that are introduced by ice flow. Detailed analysis of the crystal fabric, dust layers and chemistry may also be able to identify folded sequences. The upper 90% of the records are unaffected by ice flow and preserve a climate record with annual resolution. By detailed analysis of both cores, we anticipate that it will be possible to unravel many of the complexities that ice flow has introduced in the marine isotope stage 5e portion of the records. Deep Antarctic cores, where the marine isotope stage 5e portion occurs further from the bedrock, will also help resolve these issues. □

Comparison of oxygen isotope records from the GISP2 and GRIP Greenland ice cores

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RECENT results^{1,2} from the Greenland Ice-core Project (GRIP) Summit ice core suggest that the climate in Greenland has been remarkably stable during the Holocene, but was extremely unstable for the time period represented by the rest of the core, spanning the last two glaciations and the intervening Eemian interglacial. Here we present the complete oxygen isotope record for the Greenland Ice Sheet Project 2 (GISP2) core, drilled 28 km west of the GRIP core. We observe large, rapid climate fluctuations throughout the last glacial period, which closely match those reported for the GRIP core. However, in the bottom 10% of the cores, spanning the Eemian interglacial and the previous glaciation, there are significant differences between the two records. It is possible that ice flow may have altered the chronological sequences of the stratigraphy for the bottom part of one or both of the cores. Considerable further work will be necessary to evalu-

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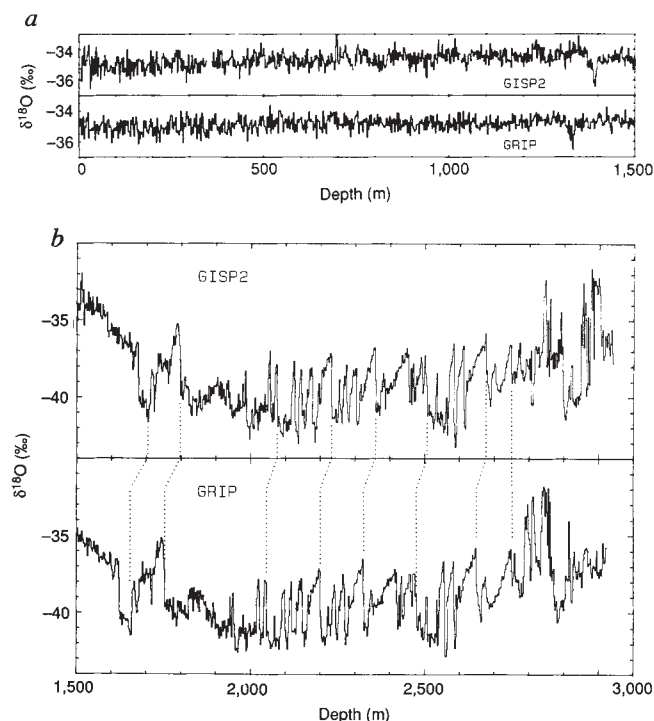


FIG. 1 GISP2 and GRIP $\delta^{18}\text{O}$ versus depth of the Greenland ice sheet at its summit area. Plot resolution is 2 m and 2.2 m for GISP2 and GRIP respectively, based on 1-m and 0.55-m data sets. The Holocene records (a) show relatively stable $\delta^{18}\text{O}$ values (mean value -34.7‰ at GISP2, -34.9‰ at GRIP). Corresponding (correlation coefficient $r=0.945$) major $\delta^{18}\text{O}$ changes of the earliest Holocene and Pleistocene (b) are marked by dotted lines down to $\sim 2,750$ m. For the record of the penultimate interglacial and beyond, below 2,750 m near bedrock, this correlation breaks down.

ate the likelihood of this, and the extent to which it will still be possible to extract meaningful climate information from the lowest sections of the cores.

On 1 July 1993 the US Greenland Ice-Sheet Project 2 completed a 5-yr ice-core drilling effort when bedrock was reached near the summit of the Greenland ice sheet (72.58° N, 38.48° W, 3,208 m above sea level, mean annual temperature -31°C). The 3,053.44-m-long, 0.132-m-diameter core was drilled with the new US Polar Ice Coring Office (PICO) electro-mechanical drill³ using *n*-butylacetate as a drilling fluid. The bottom 13.11 m of the core consists of distinctly banded brown silty ice, with silt and rock inclusions. A 1.55-m rock core was recovered from bedrock underneath the ice. About 40% of the core cross-section was sampled continuously in the field in a multi-investigator sampling program⁴. We report here (Fig. 1) the basic features of the GISP2 oxygen isotope profile, expressed as $\delta^{18}\text{O}$, the relative difference between the $^{18}\text{O}/^{16}\text{O}$ abundance ratios of the ice and standard mean ocean water (V-SMOW) expressed in per mil (‰). The $\delta^{18}\text{O}$ value of precipitation generally reflects its temperature of formation⁵ with lower $\delta^{18}\text{O}$ values corresponding to lower temperatures.

We compare the GISP2 isotope record (Fig. 1a and b) with that of the GRIP core drilled 28 km to the east at the present ice divide¹. The distance from the divide (28 km, about nine ice thicknesses) places GISP2 in a flank-flow regime. Surface and bedrock topography between the two drill sites is relatively flat⁶. As major climatically induced $\delta^{18}\text{O}$ perturbations have to occur at both sites simultaneously, a simple and direct comparison can be made between the $\delta^{18}\text{O}$ -depth profiles of the cores to evaluate the climatic significance of the observed isotope fluctuations on a summit-wide scale. This comparison by depth avoids the present uncertainties in the time-depth relationship that are to be reduced by further joint evaluation of the GISP2 and GRIP records⁷. The records are presented at 2-m (GISP2) and 2.2-m (GRIP) resolution in two parts, 0–1,500 m (Holocene) and 1,500 m depth to bedrock (mostly Pleistocene) as was previously done for the GRIP core¹.

The Holocene is a period of relatively stable climate in both cores with mean $\delta^{18}\text{O}$ values of -34.7 and -34.9 ‰ for GISP2 and GRIP respectively. The small Holocene $\delta^{18}\text{O}$ fluctuations of 1–2‰ occur too frequently to allow an unambiguous correlation between the cores. As the timescales for the cores are currently still under debate, it is only possible at this stage to compare the changes in $\delta^{18}\text{O}$ with depth between the two cores. Doing so yields virtually no correlation between the cores for the Holocene; it is likely that local differences in deposition played a major role in the small-amplitude, high-frequency changes during this period. The major $\delta^{18}\text{O}$ -temperature fluctuations in the Pleistocene are another matter. Down to 2,700 m the frequent fluctuations are quite similar in both cores, not just in general appearance, but down to the structure of the various warmer episodes. The excellent agreement of $\delta^{18}\text{O}$ down to 2,700 m between two cores drilled 28 km apart and analysed independently establishes the regional significance of the detailed isotope fluctuations for this part of the record.

The transitions between cold and warm conditions in the $\delta^{18}\text{O}$ record of the summit cores are rapid, of the order of decades (see also refs 8, 9). Corresponding changes in dust content, electrical conductivity measurement (ECM) and ice accumulation are even more abrupt^{9,10}. This excludes changes in insolation, global ice volume or oceanic water transport at depth as the cause of the rapid transitions in the $\delta^{18}\text{O}$ record, as these have all longer response times. Mayewski *et al.*¹¹ relate the rapid climate changes at GISP2 to expansion and contraction of the polar atmospheric cell, based on a factor analysis of the covariance of groups of major cations and anions found in the ice. The sequence of stadial-interstadial episodes at GRIP has recently been correlated with new, highly detailed records of sea surface temperatures from North Atlantic sediments¹². Evidently the Summit record of climate fluctuations during the glacial period down to

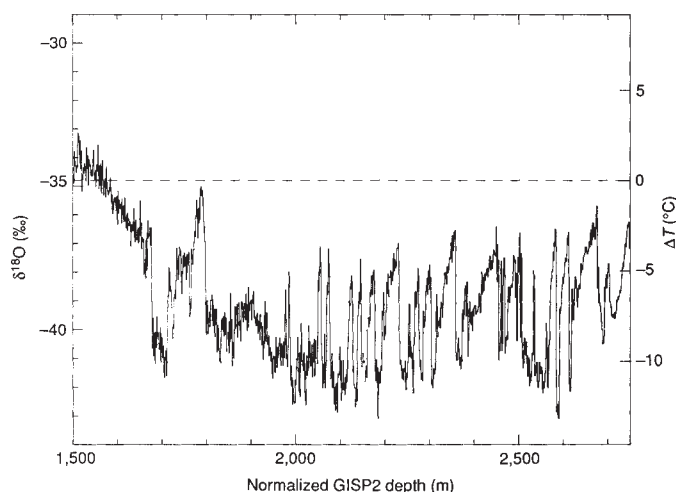


FIG. 2 Unified oxygen isotope climate record for the Summit area of the Greenland ice sheet based on the GISP2 and GRIP $\delta^{18}\text{O}$ records. Corresponding sections of the datasets were identified by using rapid $\delta^{18}\text{O}$ changes associated with Dansgaard-Oeschger interstadial events as chronostratigraphic markers. We adjusted the depth interval of the GRIP dataset between two markers to that of GISP2, and calculated simple averages over 1-m intervals. The record covers the earliest part of the Holocene, the glacial-interglacial transition and most of the last glacial. The right-hand scale shows deviations from mean Holocene temperatures (ΔT) assuming that the $\delta^{18}\text{O}$ deviation from the mean Holocene $\delta^{18}\text{O}$ of -34.8 ‰ is caused by local temperature changes with a $\delta^{18}\text{O}$ -temperature coefficient of 0.63 ‰ $^{\circ}\text{C}^{-1}$ (ref. 13).

2,700 m reflects atmospheric conditions and forcing by the North Atlantic Ocean mixed layer, and thus is of hemispheric and probably global importance.

Although a detailed timescale is still under construction⁷, a statistical comparison of the GISP2 and GRIP isotope records can be made for the 1,500–2,750 m depth interval by using the major rapid changes in $\delta^{18}\text{O}$ as chronostratigraphic markers to correct for the depth differences between the cores. An average offset between the records of 0.09‰ and a correlation coefficient $r=0.945$ for the depth-normalized records, indicating 89% common variance, confirm the excellent visual agreement between 1,500 and 2,750 m depth. Averaging the GISP2 and GRIP $\delta^{18}\text{O}$ records with 1-m resolution over the chronostratigraphically defined intervals reduces the influence of the remaining variance, which probably reflects local variability, and produces a glacial climate history for the Summit area of the Greenland ice sheet based on both ice cores (Fig. 2).

The Summit records provide a wealth of environmental information in a detail hitherto unavailable for the last glacial. Figure 2 clearly shows an unstable glacial climate. Isotope values during the last glacial switch between periods of high and low $\delta^{18}\text{O}$ values lasting several hundred to a few thousand years^{1,8,10}, except during the last glacial maximum (marine isotope stage 2) from about 1,800 to 2,050 m at GISP2. A steep rise in $\delta^{18}\text{O}$ from a low (-41 to -43 ‰) range to a high of -37 ‰ is followed, in the longer interstadials, by a gradual decrease to ~ -39 ‰ and then a precipitous drop. If we assume that the isotope values reflect central Greenland temperature and use a $\delta^{18}\text{O}$ temperature coefficient of 0.63 ‰ $^{\circ}\text{C}^{-1}$ (ref. 13), then the mean annual temperatures of the maxima occurring during the glacial for the Summit area were 3.5°C colder than the Holocene average temperature (mean $\delta^{18}\text{O}$, -34.8 ‰). Mean temperatures dropped by as much as 3°C during the longer interstadials. The $\delta^{18}\text{O}$ change from -37 ‰ to -39 ‰ during the longer interglacials corresponds to a temperature decrease of $\sim 3^{\circ}\text{C}$. Full glacial conditions were 10 – 13°C colder than during the Holocene. Close agreement between fluctuations in sea surface temperature indicated by *N. pachyderma* (s) in two North Atlantic cores and the GRIP $\delta^{18}\text{O}$ record¹² shows that the frequent, large tempera-

ture fluctuations at Summit must have been part of a larger atmospheric pattern that extended over the North Atlantic, and probably also over northwest Europe. The large number of interstadials revealed by the ice cores may be the cause of some of the confusion about the number of interstadials and their timing in northwest European climate records^{14–17} and offer an opportunity for their interpretation.

The bottom 10% of the isotope records differ significantly. Below interstadial 22 (ref. 1) (2,700 m depth at GISP2, 2,676 m at GRIP, that is 87 kyr BP in the GRIP preliminary timescale¹) layer thicknesses differ substantially; below interstadial 23 (ref. 1) (~2,750 m in both cores) the correlation between the isotope values deteriorates drastically. Taylor *et al.* report a similar breakdown of correlation between the cores for electrical conductivity measurements¹⁸. Thus, although the bottom part of both records clearly contains ice from the penultimate interglacial and beyond, the loss of a simple relationship between depth and age prevents a detailed dual-core climate reconstruction deeper than the early Wisconsin glacial. The GISP2 record of the Eemian/Sangamon interglacial period is probably affected by flow deformation and thus cannot confirm the conclusions regarding extremely high climate variability during this period that were based on the GRIP record^{1,2}.

The excellent agreement observed above a depth of 2,700 m suggests that surface climate conditions at the GISP2 and GRIP localities were similar for both cores for the entire accumulation interval. This agrees with similar peak-to-trough $\delta^{18}\text{O}$ values in the bottom part of the cores. The differences between the records must therefore result from flow deformation at one or both of the record localities. Inclined layering, first observed in visual stratigraphy at a depth of 2,678 m in the GISP2 core and noticed at 2,847 m in the GRIP core (see Taylor *et al.*¹⁸), may indicate such flow deformation. The location of the GRIP core at the ice divide presently protects the deep ice at GRIP from folding. Under different conditions of sea level, ice accumulation rate

and temperature during the glacial period, the ice divide may have moved 10–50 km, probably towards the east¹⁹. This would have placed both GISP2 and GRIP in a flank-flow regime, with the GRIP site downstream of bedrock slopes immediately to the east⁶. The greater depth at which inclined layering was reported for the GRIP core fits the predicted protection of the divide flow and suggests that the GRIP record can be interpreted climatically to a greater depth than the GISP2 record. Further work on the ice cores and the glaciology of the Summit area is needed to provide a detailed timescale for the well established glacial record down to 2,700 m, and to interpret the disturbed bottom part of the records. □

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Endogenous growth of persistently active volcanoes

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LAVA lakes and active strombolian vents have persisted at some volcanoes for periods exceeding the historic record. They liberate prodigious amounts of volatiles and thermal energy but erupt little lava, a paradox that raises questions about how volcanoes grow. Although long-lasting surface manifestations can be sustained by convective exchange of magma with deeper reservoirs, residence times of magmas beneath several basaltic volcanoes are ~10–100 years^{1,2}, indicating that where surface activity continues for more than 100–1,000 years, the reservoirs are replenished by new magma. Endogenous growth of Kilauea volcano (Hawaii) through dyke intrusion and cumulate formation is a well-understood consequence of the steady supply of mantle-derived magma^{3,4}. As we show here, inferred heat losses from the Halemaumau lava lake indicate a period of dominantly endogenous growth of Kilauea volcano during the nineteenth century. Moreover, heat losses and degassing rates for several other volcanoes, including Stromboli, also indicate cryptic influxes of magma that far exceed visible effluxes of lavas. We propose that persistent activity at Stromboli, and at other volcanoes in different tectonic settings, is evidence of endogenous growth, involving processes similar to those at Kilauea.

When Europeans first visited Kilauea volcano in 1823, they found the Halemaumau crater filled by an active lava lake. In 1880, the lake had an estimated area of $6.51 \times 10^3 \text{ m}^2$ (ref. 5). It persisted with various fluctuations until 1924 (ref. 3). Significant flank eruptions of lava took place only in 1840, 1868 and 1919–20 (refs 3, 6). The Kupaianaha lava pond which was active in the 1980s provides an analogue for the 19th century Halemaumau lava lake, although it formed part of an effusive lava system, whereas Halemaumau was located directly above a shallow reservoir at 1–2 km depth^{4,7}. We use measurements made at Kupaianaha to estimate heat losses from the Halemaumau lake. During the period 1987–8, the surface of the Kupaianaha pond, though crusted, typically remained at a temperature of 200–300 °C, corresponding to an average radiant emittance of $\sim 5 \times 10^3 \text{ W m}^{-2}$ (ref. 8), and a convective loss (forced or natural) of $\sim 2 \times 10^3 \text{ W m}^{-2}$. If these values are used for the pre-1924 Halemaumau, the inferred surface thermal losses alone imply a magma flux into the volcano of $\sim 0.6\text{--}3.0 \times 10^3 \text{ kg s}^{-1}$ (depending on the enthalpy model chosen; see Table 1 and Fig. 1). When conductive losses through the walls of the reservoir and conduit⁹, and hydrothermal losses (not easily modelled) are considered, a much larger magma influx is indicated, greatly exceeding the time-averaged surface eruption rate of $\sim 3 \times 10^2 \text{ kg s}^{-1}$ for the period¹⁰, but consistent with the observed eruption rate of $\sim 10^4 \text{ kg s}^{-1}$ from Kilauea since 1924 (refs 4, 7). This in turn provides a lower bound to the rate of supply of magma to the volcano from the mantle plume powering the Hawaiian hotspot^{4,11}.

In order to sustain the rate of heat loss from Halemaumau, we suggest that fresh magma was arriving beneath Kilauea during the 19th century at about the same rate as it has during prolonged periods of visible lava effusion in the 20th century. It follows that Kilauea was steadily 'growing'. The difference

TABLE 1 Thermal and SO₂ flux data for some persistently active volcanoes

Site/year of observation	Area (m ²)	Effective radiation temperature (°C)	Surface heat flux (MW)	Magma influx model (A) (kg s ⁻¹)	Magma influx model (B) (kg s ⁻¹)	SO ₂ flux (kg s ⁻¹)	Magma influx from SO ₂ (kg s ⁻¹)	Eruption rate (kg s ⁻¹) (period)
Halemaumau 1880 (refs 5, 6)	65,100	272	460	3,000	560	—	—	300 (1823–1923)
Kupaianaha 1987–88 (ref. 8)	2,300	272	16	110	20	—	—	—
Kilauea 1986 (refs 23, 26)	—	—	—	—	—	13.5 ± 4.6	14,000	~10,000 (Observed recent output)
Stromboli 1992 (P. Allard)	—	—	—	—	—	9.25	9,300	0.3 (?Since antiquity)
Etna 1975–1987 (ref. 31)	—	—	—	—	—	46.3 ± 9.0	46,000	~1,000 (1759–1974)
Nyiragongo 1959 (ref. 18)	13,125	650	620	4,000	750	12.6	13,000	—
Erta 'Ale 1973 (ref. 19)	7,600	573	260	1,700	320	0.58	580	—
1986 (ref. 32)	30,600	176	100	670	130	—	—	—
Masaya 1979 (ref. 21)	—	—	—	—	—	13.9	25,000	10 (since 16th C)
Erebus 1985 (ref. 22)	35,100	137	84	540	100	—	—	—
1987 (ref. 32)	—	—	—	—	—	0.59	590	—
Láscar 1987 (ref. 32)	34,200	171	110	730	140	—	—	450
1989 (ref. 33)	—	—	—	—	—	27.5	28,000	(1984–1993)

Thermal flux estimates are made mostly from satellite observations³². Effective radiation temperature is defined as $T_e = (E/\sigma)^{0.25}$, where E is the radiant emittance and σ is the Stefan–Boltzmann constant. Heat fluxes combine radiative and convective losses estimated for the given T_e , but exclude sub-surface conductive and hydrothermal losses. Magma influxes calculated from heat losses are given for models (A) and (B) of Fig. 1. SO₂ fluxes were determined by correlation spectrometer (COSPEC), except for Erta 'Ale and Nyiragongo which were determined by chemical methods. Errors in thermal fluxes are discussed in ref. 32. SO₂ fluxes are reported directly from cited references; errors are often not given. Magma influxes based on SO₂ data were determined for 0.05 wt% degassable sulphur in melt, as at Kilauea, except for Masaya, for which petrological data were available. Eruption rate for Láscar is dominated by the single 1993 eruption volume.

between the inferred rate of magma input and the 19th century surface output is a measure of the rate of endogenous growth.

How does endogenous growth take place? One mechanism is dyke intrusion. When Halemaumau was active, the lava lake exhibited occasional abrupt decreases in level, without flank eruptions. These events were sufficiently striking to provoke contemporary enquiries⁶, and indicate the intrusion of dykes into Kilauea's rift system. Some of the 'missing' lava may have been erupted on the sea floor, though there is no historical evidence for this³⁴. During the period 1956–83, ~35% of Kilauea's input of ~10⁴ kg s⁻¹ was extruded; the remainder was stored within the eastern and southwestern rift zones¹². Dyke intrusion and rift-zone widening are accommodated by seaward displacement of the south flank of the island of Hawaii along the prominent *pali* faults³ (Fig. 2).

A second mechanism of endogenous growth is the formation of cumulates, which can help drive convection in the overlying reservoir by means of crystallization and cooling. Walker suggested the progressive growth of layers of solidifying magma that maintain themselves at a level of neutral buoyancy¹³, an hypothesis which is supported by seismic data indicating the existence of major cumulate formations in the lower crust beneath the Hawaiian islands¹⁴. These large magmatic increments beneath the volcanoes are partly compensated by bending of the lithosphere in response to loading, leading to rapid subsidence of the islands¹⁵.

Many other basaltic volcanoes around the world display persistent magmatic activity. Although differences in scale and tectonic setting may suggest that they have little in common with Kilauea, there are some surprising similarities. Stromboli, the prime example, has been active for millenia, prompting several enquiries into the nature of its persistent activity (see, for example, refs 16 and 17). Etna has also been active throughout

history, exhibiting strombolian activity and intermittent lava effusion. Its average eruption rate between 1759 and 1974 was ~10³ kg s⁻¹ (ref. 11). Both Nyiragongo (Zaire)¹⁸ and Erta 'Ale (Ethiopia)^{19,20} have probably had active lava lakes throughout historic times. Nyiragongo's lake was contained in a deep crater until this was catastrophically breached in 1977. Only minor lava flow effusion has been noted at Erta 'Ale. At Masaya (Nicaragua), a lava lake has been present intermittently over the past 450 years, although the time-averaged eruption rate is only ~10 kg s⁻¹ (ref. 21). Mt Erebus (Antarctica) contains a small, persistent lava lake of anorthoclase phonolite and has exhibited frequent minor strombolian eruptions since its discovery in the 19th century²².

We can use observed thermal and/or SO₂ degassing fluxes to constrain influxes of hot volatile-rich magma into sub-surface reservoirs for these volcanoes (Table 1). Magma fluxes are derived from measured SO₂ fluxes by assuming 0.05 wt% degassable sulphur in parental magmas²³. During the sustained Pu'u O'o (Kilauea) eruption of the 1980s, the observed rate of surface lava effusion was close to the long-term rate of magma supply. Good correlations between the magma flux inferred from measured gas fluxes, and the observed rate of eruption confirms that gas flux provides a useful proxy measure of magma flux²³. The discrepancies between magma fluxes derived by thermal and gas flux methods are not surprising: thermal modelling is difficult, and gas flux data are sparse and of variable quality. More important than the accuracy of individual estimates is their wider significance: fresh, volatile-rich magma from depth must continuously reach the surface, where it degasses and cools before returning to the reservoir. Magma convection within the conduit connecting the reservoir to the surface is thought to be driven by the increase in melt density that accompanies volatile exsolution^{24,25}.

Stromboli's edifice is much smaller than Kilauea, and its time-averaged eruption rate is a mere 0.3 kg s^{-1} (refs 16, 17), but its average SO_2 flux of $\sim 10 \text{ kg s}^{-1}$ (P. Allard, personal communication) is similar to that of Pu'u O'o²⁶, implying a comparable magma flux. Giberti *et al.*⁹ calculated that $\sim 200 \text{ kg s}^{-1}$ of magma must be supplied to the upper parts of Stromboli's edifice, to sustain heat losses through degassing and conduction, and to account for the inferred gas flux. They suggested that this supply might come either from a deep mantle source or from convective overturn of a large magma chamber; however, uranium-series isotopic disequilibria studies for both Stromboli and Etna suggest that the residence time for shallow magma is only several tens of years^{1,2} indicating that their persistent surface manifestations cannot be sustained by convective circulation between surface vents and un-replenished reservoirs. Even a conservative interpretation of the thermal and gas flux data therefore implies that new magma must be supplied to these and similar volcanoes at rates of hundreds or thousands of kilograms per second. Whenever the inferred steady-state supply of mantle-derived magma exceeds the rate of eruption, formation of sub- and/or intra-volcanic intrusive complexes is indicated.

It follows that persistently active volcanoes are growing endogenously at far greater rates than their modest surface eruptions may suggest. At Kilauea, magmatic increments have been evident at the surface in recent decades, when lava has been steadily streaming into the sea, but were mostly cryptic during the last century when the lava lake at Halemaumau was the primary manifestation of magmatic activity. At Stromboli, magmatic increments appear to have been dominantly cryptic throughout history, whereas Etna has exhibited both prolonged periods of cryptic activity and occasional outpourings of lava. The typical SO_2 flux for Masaya (14 kg s^{-1} in 1979) has been interpreted as requiring a magma flux of $2.5 \times 10^4 \text{ kg s}^{-1}$ (ref. 21), which is almost entirely cryptic.

As Kilauea demonstrates, rapid endogenous growth of volcanoes can be accommodated by minor intrusions accompanied by edifice deformation, and by formation of cumulate complexes at deeper levels. About 20% of Stromboli's edifice is composed

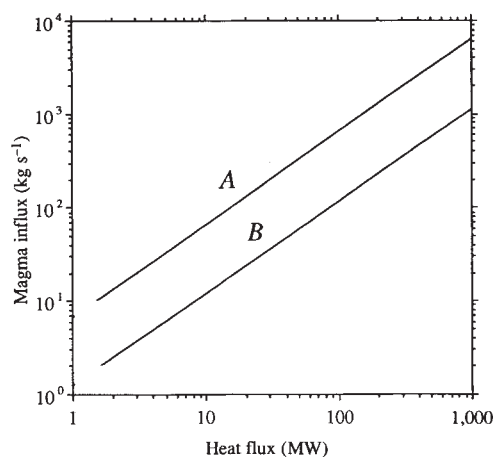


FIG. 1 Influxes of magma required to balance thermal losses from a reservoir at constant temperature and volume. Enthalpies are calculated for a parental magma that cools $\Delta T(^{\circ}\text{C})$ and crystallizes a mass fraction Δf before becoming thermally isolated from the reservoir. Assuming that all the heat extracted is transferred to the reservoir, the mass influx of parental magma is given by $q/(L\Delta f + c\Delta T)$ where q is the thermal loss (W), L is the latent heat of fusion, and c is the specific heat capacity. Two enthalpy models are given, corresponding to dykes and cumulates, respectively: (A), $\Delta T = 50^{\circ}\text{C}$, $\Delta f = 0.25$; (B), $\Delta T = 400^{\circ}\text{C}$, $\Delta f = 1$ (L taken as $4.2 \times 10^6 \text{ J kg}^{-1}$; c as $10^3 \text{ J kg}^{-1} \text{ K}^{-1}$).

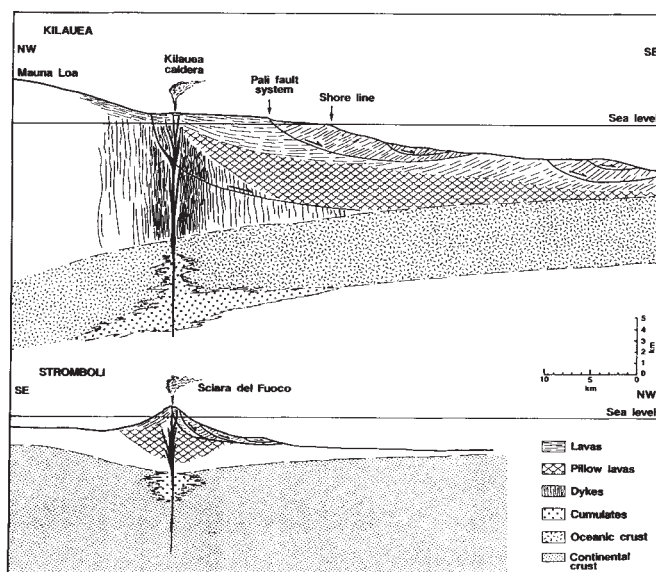


FIG. 2 Cross-sections of Kilauea (top) and Stromboli (bottom) to same scale, showing postulated overall similarities in two-dimensional internal structure, and mode of growth by flank displacement and cumulate formation. Kilauea section from ref. 13, based on seismic data; Stromboli section schematic, based on maps and sections in refs 27 and 28, and sources cited therein. The rotational faults illustrated are based on "shovel-shaped faults" first proposed by Rittman²⁷; sub-volcanic cumulates are inferred from geochemical evidence and sections in ref. 28. Lithospheric flexure illustrated is conjectural. Stromboli's geodynamic setting is poorly understood, but the volcano is thought to be constructed on continental crust 15–20 km thick.

of minor dykes and sills²⁷. Over the last $\sim 10^4$ yr, its active vents have been located within the Sciara del Fuoco on the northwestern flank of the volcano, an amphitheatre-like sector bounded by faults²⁸. We suggest therefore that Stromboli's structure and mode of growth resemble that of Kilauea, and that its endogenous growth is similarly accommodated, in part, by downward and outward movements along extensional faults that provide a stress regime favourable for minor intrusions. Loading of the lithosphere by formation of cumulate complexes in crustal reservoirs may also cause flexure similar to that observed beneath the Hawaiian islands¹⁴ (Fig. 2).

Little is known about the sub-surface structures of volcanoes such as Nyiragongo, Erta 'Ale and Erebus, but all three are located in extensional environments likely to promote rifting and dyking²⁹. Whatever the form of volcano, continued loading will lead to internal deformation, spreading and possible flank failure, as at Etna³⁰. Lava lakes and persistent strombolian activity represent the most tangible evidence of endogenous activity, but other quiescent volcanic manifestations are also consistent with long-term growth. For example, sustained power outputs of aqueous crater lakes (such as Ruapehu, New Zealand) and lava domes (such as Lascar, north Chile) typically exceed 10^8 W . Independent quantification of the rates of endogenous growth will be difficult, and will require geophysical and geodetic monitoring. Shallow-level intrusions may be detectable through ground deformation and gravity changes, but deeper-level phenomena would be hard to detect.

More fundamental questions concern the factors that control the ratios of intrusive to extrusive volcanic activity. These must involve complex interactions between magma density and lithospheric subsidence rates. If efficient convective circulation allows extensive degassing of magma from a persistently active vent, then the resulting density increase will inhibit eruption of lavas and promote cumulate formation at depth. If lithospheric subsidence is rapid, the magma column may always be near

the surface, favouring lava eruptions; if subsidence is slow, the volcanic edifice may accumulate to such a height that ultimately only the lowest-density magmas can erupt. □

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Deep seismic reflection evidence for continental underthrusting beneath southern Tibet

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THE Himalaya and adjacent Tibetan plateau, constituting Earth's largest region of elevated topography and anomalously thick crust, formed as a consequence of Cenozoic collision between India and Asia—itself considered the archetypal continent–continent collision^{1–3}. Here we report the first results from an attempt to image the structure of the crust beneath this region using deep seismic reflection profiling. Our ~100-km-long profile, acquired in the Tethyan Himalaya, shows a mid-crustal reflection that probably marks the active thrust fault along which the Indian plate is underthrusting southern Tibet; upper-crustal reflections with geometries suggestive of large-scale structural imbrication of the upper crust; and Moho reflections from the base of the double-normal-thickness crust underlying the region. These results lend substantial support to the view that crustal thickening beneath southernmost Tibet was accomplished by wholesale underthrusting of Indian continental crust beneath the structurally imbricated upper crust comprising the Tethyan Himalaya.

The deep seismic reflection data were collected by Project INDEPTH (International Deep Profiling of Tibet and the Himalaya), which is a cooperative programme between the Chinese Academy of Geological Sciences of the Ministry of Geology and Mineral Resources of China (MGMR) and Earth scientists from several universities in the United States. The profile was sited within the southern part of the Yadong–Gulu rift in southern

Tibet (Fig. 1). The Yadong–Gulu rift is one of a series of north–south trending Quaternary grabens that transect the Himalaya and southern portion of the Tibetan Plateau⁴. The portion of the Yadong–Gulu rift containing the INDEPTH survey lies within the Tethyan Himalaya, largely comprising Palaeozoic and Mesozoic miogeoclinal strata that were deposited on the north-facing continental margin of India and subsequently folded and thrust southward during Neogene collision between India and Asia^{5,6}. Collision-related compressive deformation within the Tethyan Himalaya was apparently followed by an episode of north–south extension, manifest principally by the development of the South Tibetan Detachment (STD), a major low-angle north-dipping ductile normal fault that now forms the boundary

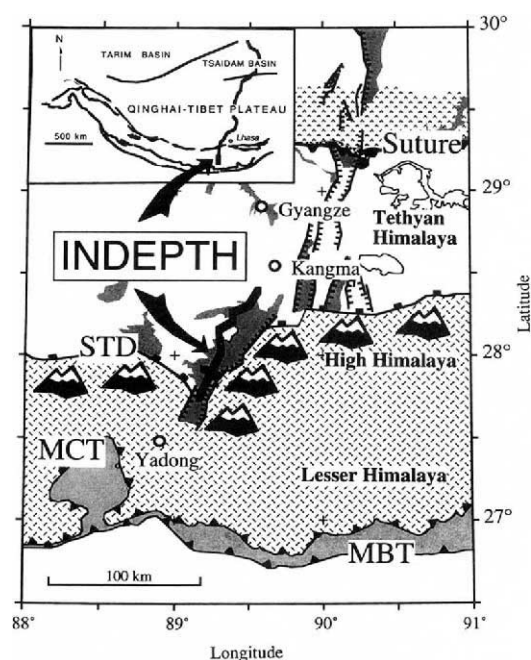


FIG. 1 Generalized geological map showing location of INDEPTH pilot deep seismic profile. MBT, Main Boundary Thrust; MCT, Main Central Thrust; STD, South Tibetan Detachment; Suture, Indus/Tsangpo suture; schematic mountains, crest of High Himalaya.

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between the Tethyan Himalayan sedimentary strata and High Himalayan crystalline sheet to the south⁷. Other extensional structures presumed to have formed during the same episode include brittle normal faults that cut and/or reactivate thrust faults within the Tethyan Himalaya, and a major top-to-the-north ductile shear zone that frames the crystalline Kangmar Dome, which lies within the Tethyan Himalaya immediately north of the INDEPTH profile⁸. Geochronological data demonstrate that the STD formed during convergence between India and Asia, and while active thrust faulting was occurring at lower altitude on the south flank of the Himalaya⁷. Intracrustal earthquakes with thrust-type first motions, occurring beneath the south flank of the Himalaya, indicate continuing convergence between India and southern Tibet⁹.

The INDEPTH seismic reflection profile is a nominal 15-fold common-midpoint (CMP) stacked section acquired with explosive sources, 50-m receiver-group spacing and 50-s listening time (Fig. 2). Processing includes elevation statics, pre-stack gain balance, bandpass filter, deconvolution, normal-moveout (NMO) correction, CMP stack, post-NMO mute, post-stack gain balance and display. Long-offset and off-line seismic data were also acquired during the field program with portable REFTEK seismographs.

The most prominent laterally extensive feature visible on the INDEPTH seismic profile is a 'mid-crustal' reflection that extends across the entire section, dipping gently to the north (~ 9 – 13 s two-way travel time, TWTT, Fig. 2). Sino-French refraction data collected in the Tethyan Himalaya, in the early 1980s, indicate that the mean crustal velocity above this horizon is in the range 6.0 – 6.4 km s⁻¹ (ref. 10). Using this range of velocities to convert echo-time to depth, and projecting the reflection profile onto a north-south section, yields an average northward dip for this feature of $\sim 9 \pm 2^\circ$. At the southern end of the profile the reflection is at $\sim 28 \pm 1$ km depth and at the northern end $\sim 40 \pm 2$ km depth. Wide-angle reflections recorded by REFTEKs deployed north of the CMP profile indicate that this horizon extends in the subsurface at least 30 km beyond the north end of the profile.

Comparison with existing earthquake and geological data suggests that this reflection marks the thrust fault along which India is presently underthrusting southern Tibet—termed here the Main Himalayan Thrust (MHT). Figure 3 shows the spatial relationship of the INDEPTH profile to existing seismological constraints on the position of the MHT⁹; the reflection data have been projected onto a north-south profile, and positioned at the appropriate distance north of the reference position for the earthquake data. Although the positioning of the reflection profile is approximate, a straightforward interpretation is that the midcrustal horizon imaged on the profile is the northern continuation of the active India-Asia thrust (MHT). Balanced cross sections across the eastern Nepal Himalaya, constructed from surface geological data, also imply a depth to the MHT beneath the High Himalaya of ~ 30 km^{11,12}—in essential agreement with the depth to the interpreted MHT reflection at the

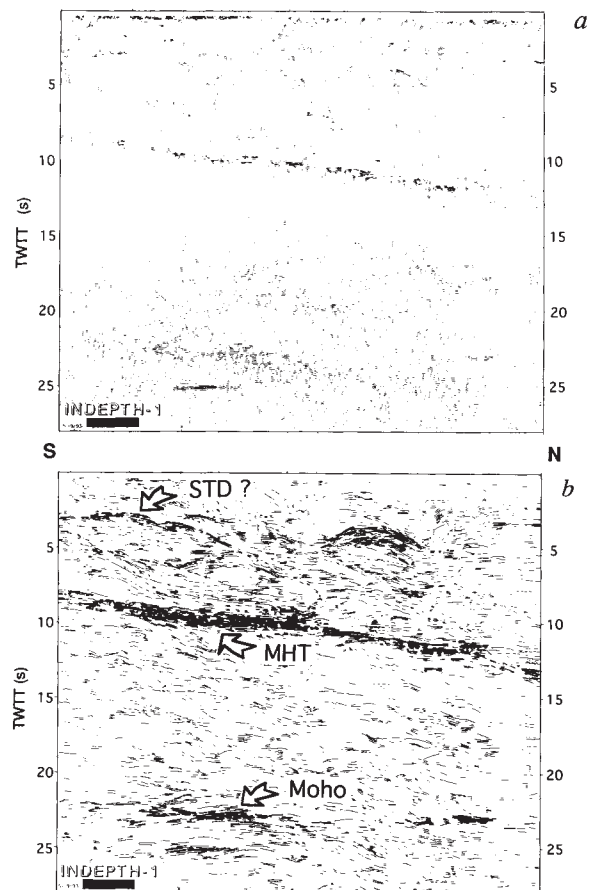


FIG. 2 *a*, upper 30 s of INDEPTH pilot deep seismic profile (unmigrated). Display is $\sim 1:1$ (that is, on the same distance scale horizontally and vertically) assuming an average crustal velocity of 6 km s⁻¹. *b*, Generalized line drawing showing interpretation of principal features. MHT, Main Himalayan Thrust; STD, South Tibetan Detachment; Moho, interpreted base of Indian continental crust underthrusting southern Tibet. Scale bar (lower left) is 10 km.

south end of the INDEPTH profile. The reflection data imply that Indian continental crust in the footwall of the MHT dips gently to the north beneath southern Tibet, and extends at least as far north as the middle of the Tethyan Himalaya—some 200 km north of the Himalayan thrust front (MBT, Fig. 1). This is substantially north of its last seismologically constrained position, but well within the ~ 440 km of total convergence between India and southern Tibet that has been estimated from palaeomagnetic data to have occurred since the initiation of

FIG. 3 Spatial relationship of INDEPTH seismic profile on the north side of the Himalaya to well located thrust-type earthquakes defining the active India-Asia decollement beneath the south side of the Himalaya (modified from Fig. 16 in ref. 6). MBT, MHT, Moho, same as in Figs 1 and 2. Grey line, approximate Moho beneath Himalaya interpreted from gravity data¹⁵.

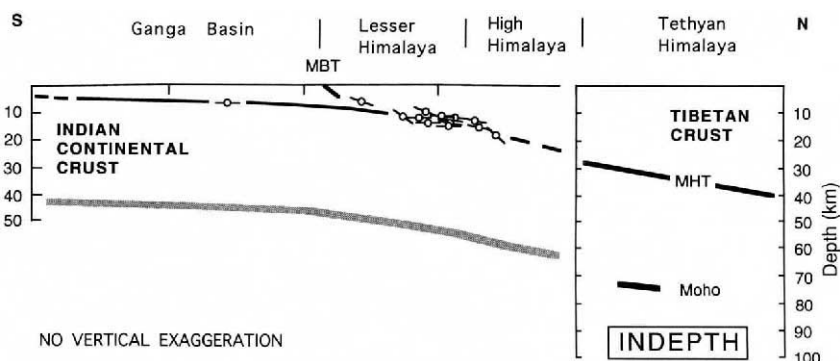
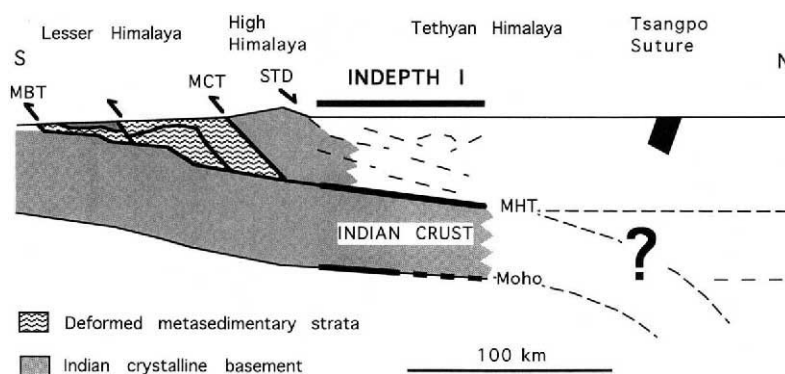


FIG. 4 Schematic crustal cross-section of the Himalaya and the southern Tibetan Plateau showing location of INDEPTH seismic profile and present limit of knowledge of large-scale crustal structure ($\sim 1:1$). Upper-crustal structure south of the crest of the Himalaya is generalized from ref. 11. MBT, MCT, MHT, STD, same as in Figs 1 and 2.



Himalayan collision (~ 45 Myr ago)¹³. As the potential error in the palaeomagnetic estimate is comparable in magnitude to the estimate itself, a basic goal of deep profiling in the region is to determine the actual northward extent of Indian crust beneath the Plateau.

Large-scale displays of the INDEPTH profile show that the crust above the MHT is characterized by a number of arched and north-dipping reflections, of generally lower amplitude than the MHT reflection. The latter generally appear to flatten at or above the MHT. Although specific interpretation of these reflections awaits migration of the data and correlation with mapped surface structures, the general aspect of the reflectivity is consistent with the view developed from surface geology that the upper crust beneath the Tethyan Himalaya is imbricated on a large scale⁶. A particularly prominent reflection visible in the upper crust at the south end of the section, which dips to the north and appears to merge with the MHT, is in approximately the right position to originate from the South Tibetan Detachment (Fig. 2). Further surface geological investigation in the vicinity of the south end of the INDEPTH profile is required to test this correlation.

Finally, a notable feature of the seismic section is the occurrence of coherent reflections at echo-times which, in typical continental areas, would correspond to the upper mantle. Particularly prominent are two sets of reflections visible on the southern part of the profile—an upper band between approximately 23 and 24 s TWTT, and a lower discrete reflection at 25.2 s (Fig. 2). The reflections at 23–24 s almost certainly originate from the Moho at the base of the Indian continental crust underthrusting southern Tibet (Fig. 4): Sino-French refraction data¹⁰ indicate that the crust in the general vicinity of the INDEPTH survey has a full thickness of ~ 75 km and a mean compressional velocity of ~ 6.3 km s⁻¹, yielding an expected vertical incidence echo-time for the Moho of 23.8 s. Although the Moho reflections are most prominent on the southern part of the profile, they do appear to extend intermittently across the entire profile. Analysis of the reflection data together with daily noise recordings suggests that the large lateral variation in the apparent amplitude of these reflections is a consequence of vary-

ing ambient noise levels along the profile—much of which was wind-generated. Interpretation of the apparently deeper reflection at 25.2 s is problematic. A short cross-line that was acquired to constrain the geometry of the deep reflections indicates that this reflection has a substantial component of eastward dip. Migration, in turn, indicates that its source lies within the lower-crust, west of the profile. The interpreted Moho reflections, in contrast, lie in the plane of the profile.

The INDEPTH profile, viewed in combination with existing geological and seismological constraints, suggests that an essentially complete section of Indian continental crust has been underthrust beneath the southern Tethyan Himalaya. At the location where the Moho reflections are most prominent on the profile, the thickness of interpreted Indian crust between the MHT and Moho is ~ 43 km. This thickness is not resolvably different from the ~ 40 km thickness that has previously been determined for the undeformed crust of northeastern India, south of the Ganga basin, based on combined interpretation of seismic refraction and gravity data¹⁴. Surface geological data imply that the crust above the MHT is composed principally of thrust-imbricated sedimentary and crystalline strata that were detached from the leading edge of India early in the Himalayan collision^{5,6}. The reflection data, in turn, imply that the crust below the MHT is essentially intact Indian continental crust that was underthrust beneath this imbricate assemblage as a consequence of continuing convergence. Although the INDEPTH data do not directly constrain the shape of the Moho south of the profile, the implied regional dip of the Moho between the Himalayan foreland and southern edge of the Tethyan Himalaya is $\sim 15^\circ$ towards the north, which is compatible with previous estimates based on gravity modelling¹⁵. Dipping reflection segments visible on the seismic profile within the interpreted underthrust Indian crust might be manifestations of some internal strain; however, the crustal thickness estimate for the lower plate militates against substantial distributed shortening (thickening) of the underthrust Indian crust. Similarly, no substantial fault offsets of the underthrust Indian Moho are evident on the INDEPTH profile, though such offsets might exist to the north or south¹⁶. □

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Localized *hedgehog* activity controls spatial limits of *wingless* transcription in the *Drosophila* embryo

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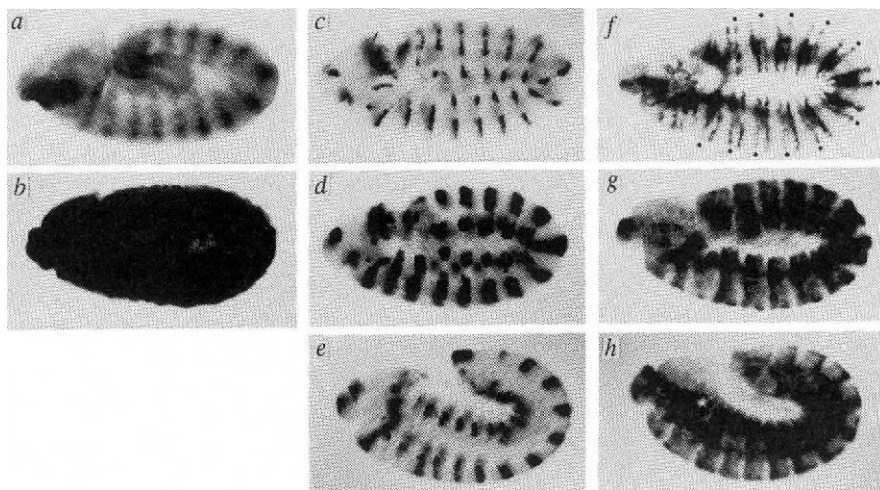
CELL patterning in the body segments of the *Drosophila* embryo requires activity of the segment polarity genes, a molecularly heterogeneous group defined by a generic mutant phenotype¹. Two of these genes, *wingless* (*wg*) and *hedgehog* (*hh*), encode proteins that enter the secretory pathway²⁻⁴, implicating them as signals that instruct the fates of neighbouring cells⁵. Genetic analysis has identified *wg* transcription as one of the targets of *hh* activity^{6,7} and it has been suggested that the spatial control of *wg* expression depends on the limited range of the *hh* signal and the differential competence of responding cells⁸. I have tested this model by driving ubiquitous expression of the *hh* gene using the Hsp70 promoter. Here I report that, as predicted, this causes the ectopic activation of *wg* in only a subset of the cells of each parasegment. Using another target of *hh* activity as a probe, I demonstrate that the competence of cells to express *wg* is independent of their ability to receive the *hh* signal. Finally, I show that *wg* activation requires the function of the segment polarity gene *fused*, suggesting that the putative *hh* signal is transduced by the serine/threonine kinase that *fused* encodes.

The subdivision of the *Drosophila* embryo into parasegments⁹ is accompanied by activation of the segment polarity genes *wingless* and *hedgehog* in stripes of cells that flank the parasegment

boundaries^{4,10,13}. Both genes encode putative signalling molecules^{4,11-14} and immunolocalization studies support a role for each protein in cell-cell signalling^{2,3,15}. The best characterized targets of *hh* activity are the *wg* and *patched* (*ptc*) genes: in *hh* mutant embryos, expression of both genes disappears from ectodermal cells after the completion of gastrulation^{6,7}. Although expression of *wg* is restricted to cells immediately anterior to those expressing *hh*, *ptc* is transcribed in cells on either side of each *hh* domain^{16,17}; thus the *hh* signal elicits distinct responses in cells according to their position within the parasegment. To account for these differential responses, it has been proposed that each parasegment is subdivided into groups of non-equivalent cells⁸. According to this model, the potential to respond to *hh* by expressing *wg* is restricted to cells in the posterior half of the parasegment, and only a subset of such 'wg-competent' cells realize this potential because of the spatially restricted nature of the *hh* signal. It follows that ubiquitous expression of *hh* should result in ectopic transcription of *wg*, but only in the posterior part of each parasegment that is wg-competent.

To investigate this hypothesis I generated transgenic HS-*hh* flies that carry a copy of the *hh* complementary DNA fused downstream of the Hsp70 promoter. Induction of ubiquitous *hh* expression from this transgene (Fig. 1b) at any time between 3 and 7 hours after fertilization leads to the ectopic activation of *wg* in cells anterior to each normal *wg* domain (Fig. 1c-e). Although the restricted nature of this ectopic induction is consistent with the competence model⁸, it is also possible that more anterior cells are unresponsive simply because they do not express an appropriate *hh* receptor. Yet when *hh* is ubiquitously expressed, *ptc* is activated throughout each parasegment, except in cells in which its transcription is always repressed by the activity of *engrailed* (Fig. 1f-h); thus cells in the anterior half of each parasegment are responsive to ectopically expressed *hh*. That this response varies with position in the parasegment supports the proposal⁸ that different regions of each parasegment

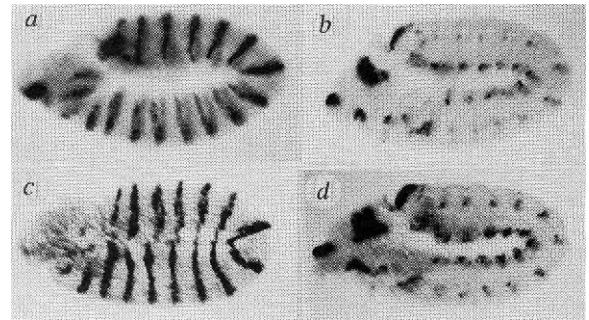
FIG. 1. Induction and consequences of ubiquitous *hh* expression using the *hsp70* promoter. Distribution of Hh protein in a stage-10 wild-type embryo (a) and a HS-*hh* embryo fixed 15 min after a 30-min heat shock (b). In normal embryos, Hh is associated with the cell membrane in discrete patches and is distributed over a broader stripe of cells than those in which it is transcribed¹⁵. After induction of the HS-*hh* transgene, the Hh protein is ubiquitously distributed (b). c, Distribution of *wg* transcript in a stage-11 wild-type embryo: expression in each parasegment is restricted to narrow stripes of cells at the posterior boundaries, interrupted by lateral repression. d, Distribution of *wg* transcript in a stage 11 HS-*hh* embryo, heat-shocked for 30 min at 4±1 and 6±1 hours after fertilization and fixed 15 min later. Each stripe of cells expressing *wg* at the parasegment boundary is considerably broader than in wild type, because of the ectopic activation of transcription in cells anterior to the normal domain of *wg* transcription. This ectopic activation is stable, as shown by its persistence in a similarly treated embryo fixed 90 min after the second heat shock (e). f, Distribution of *ptc* transcript in a stage-11 wild-type embryo. In contrast to its earlier distribution in broad stripes^{16,17}, expression of *ptc* by this stage of development is limited to two narrow stripes of cells in each parasegment that flank the cells expressing *en* and *hh*. The less intense of each of these domains (indicated by dots) corresponds to the cells that also express *wg*. g, Expression of *ptc* in a HS-*hh* embryo treated identically to that in d. Like *wg*, expression of *ptc* is ectopically activated by ubiquitous *hh*, but in this case activation extends throughout each parasegment with only those cells in which transcription is repressed by endogenous *En* activity failing to express the gene. This ectopic activation is also stable, as shown by its persistence in a similarly treated embryo fixed 90 min after the second heat shock (h).



METHODS. A *Sma*I-*Eco*RV fragment, containing the entire *hh* ORF, was purified from the plasmid *chh12* (ref. 13) and ligated with *Hpa*I-digested transformation vector pCaSpeRhS (ref. 26). The recombinant plasmid, pHS*hh* containing the *hh* ORF in the correct orientation relative to the heat-shock promoter, was selected after restriction enzyme analysis of miniprep DNA from transformed colonies and used to transform *Drosophila* embryos using standard microinjection procedures²⁷. For heat-shock treatments, embryos were collected from appropriate parents over 2-h intervals, transferred to thin layers of 1% agarose on glass microscope slides and incubated in a plastic Petri dish floating in a water bath at 37 °C for 30-min intervals. After heat treatment, embryos were returned to 25 °C before being fixed for staining with mouse polyclonal anti-Hh antisera¹⁵ or for *in situ* hybridization with digoxigenin-labelled single-stranded *wg* or *ptc* RNA probes as previously described⁸.

FIG. 2 a, Expression of *hh* transcript in a late stage-10 embryo that lacks wild-type *fu* activity. The pattern of expression is essentially the same as in wild-type embryos at the same stage^{4,11-13}. b, Expression of *wg* in a stage-11 embryo that lacks wild-type *fu* activity: note the absence of the stripes of expression in each parasegment typical of wild-type embryos (compare with Fig. 1c); transcript persists in the procephalic lobe, labrum, foregut and hindgut primordia and in neuroblasts and dorsal patches in each parasegment, sites of expression that are independent of *hh* function. c, Distribution of En protein in a HS-*hh* stage-11 embryo fixed 15 min after two 30-min heat shocks at 4 ± 1 and 6 ± 1 after fertilization. Expression of En is restricted to cells at the anterior margin of each parasegment as in wild-type²⁸ and there is no ectopic expression of the protein. d, Expression of *wg* in a stage-11 embryo that lacks wild-type *fu* activity and carries the HS-*hh* construct following heat shocks at 4 ± 1 and 6 ± 1 h after fertilization: note the absence of broad stripes of expression in each parasegment as seen following induction of ubiquitous *hh* expression in a wild-type background (compare with Fig. 1d). In about 10% of such embryos, expression is observed in small patches of ectodermal cells in a few parasegments; this is probably attributable to the residual *fu* activity contributed by the *fu* alleles used.

METHODS. Because *fu* is expressed both maternally and zygotically, it is necessary to eliminate its activity from the female parents as well as from the zygotes to be analysed. Complete loss of *fu* activity, however, results in lethality; therefore, two hypomorphic alleles, which have resi-



dual *fu* activity, were used in this analysis. Females of the genotype *fu*¹/*fu*^{v22} (S. M. Pinchin, unpublished results) were crossed to males of the genotype *fu*²; HS-*hh* M2/TM3, *hblacZ*. All of the progeny of this cross lack wild-type *fu* activity but in half of them *hh* expression can be induced independently of its endogenous control. Embryos were subjected to heat shocks as described. After fixation, embryos were hybridized with digoxigenin-labelled single-stranded *wg* and *lacZ* probes and those carrying the HS-*hh* construct were identified by the absence of *lacZ* transcript.

have differing transcriptional competences.

Previous studies have shown that the effects of *hh* mutants on *wg* transcription can be suppressed by eliminating the activity of *ptc* (refs 7, 8): because *ptc* normally functions to repress *wg* transcription^{7,18} but Ptc protein is expressed both in cells that express *wg* as well as in those in which *wg* transcription is normally repressed^{8,15}, it has been postulated that *hh* acts by somehow antagonizing the activity of *ptc* (ref. 8). In this case the effect of ectopic expression of *hh* in *ptc* mutant embryos should be indistinguishable from that of *ptc* mutants alone; consistent with this, the expression of *wg* in heat-shocked *ptc*; HS-*hh* embryos is identical to that of their non-heat-shocked siblings (data not shown).

Like *hh*, activity of the *fused* (*fu*) gene¹⁹, which encodes a putative serine/threonine kinase²⁰, is required for maintenance of *wg* transcription at each parasegmental boundary²¹.

Transcription of *hh*, by contrast, persists for longer in embryos lacking wild-type *fu* activity (Fig. 2a), suggesting that *fu* may act 'downstream' of *hh* to regulate *wg* transcription. To investigate this possibility further, I induced ubiquitous *hh* expression in embryos lacking wild-type *fu* activity (see legend to Fig. 2). The distribution of *wg* transcript observed in such embryos is usually indistinguishable from that in their non-heat-shocked sibs. There is no expression of *wg* in the ventral ectodermal cells of each parasegment, though transcript is still found in other cells where *wg* expression is independent of *hh* function⁷ (see Fig. 2d). Thus in the absence of *fu*, *hh* can neither maintain nor induce ectopic *wg* transcription. It follows that *fu* is required for the activity of *hh*, suggesting that, like other well characterized signalling factors, the putative *hh*-encoded signal is transduced by a serine/threonine kinase-dependent pathway.

In embryos homozygous for loss-of-function alleles of *hh*,

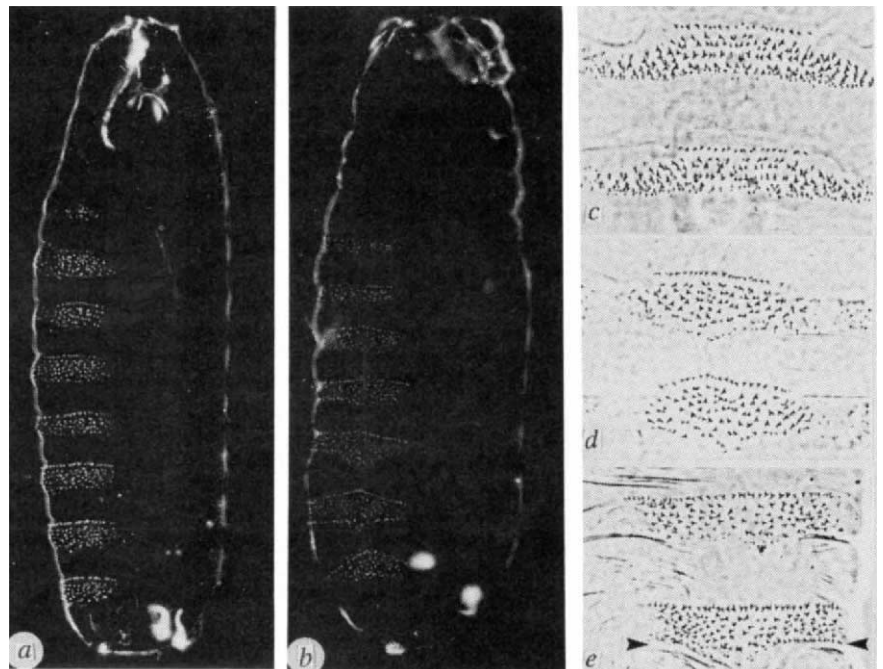


FIG. 3 Developmental consequences of the ubiquitous expression of *hh*. a, Cuticular preparation of a pharate HS-*hh* larva that was heat-shocked at 4 ± 1 and 6 ± 1 h after fertilization. In contrast to wild type, each ventral denticle belt in the abdominal segments is rectangular rather than trapezoidal. b, Cuticular preparation of larva homozygous for a loss-of-function *ptc* mutation; note the similar modification of the shape of each denticle belt. In both cases this appears to be due to a change in identity of the cells underlying the posterior part of each belt, such that they now secrete denticles characteristic of the more anterior rows 2 and 3. This can be seen in more detail in d for HS-*hh* and in e for *ptc*; c, equivalent region of a wild-type larva for comparison. The HS-*hh* phenotype is similar, though not identical to that typical of *ptc* mutations; in the latter an ectopic row 1 with reversed polarity is formed at the posterior of most belts, indicated by arrowheads in e.

expression of *en* is lost from ectodermal cells after gastrulation²², indicating that the activity of *hh*, like that of *wg*, is also required for the maintenance of *en* transcription. Although ubiquitous expression of *wg* results in the ectopic activation of *en* (ref. 23), the distribution of En protein is unaltered in heat-shocked HS-*hh* embryos (Fig. 2c). Thus, although necessary for the maintenance of *en*, expression of *hh* is not sufficient for its activation.

The changes in the patterns of *ptc* and *wg* transcription induced in HS-*hh* embryos are very similar to those observed in embryos that lack activity of *ptc* (refs 6, 18). To determine whether these changes have similar phenotypic consequences, heat-shocked HS-*hh* embryos were allowed to complete development and their cuticular patterns analysed. Embryos treated within the first seven hours of development complete embryogenesis but most fail to hatch. The pharate larvae display a striking alteration in the cuticular pattern of each segment: the ventral denticles characteristic of the posterior part of each belt are eliminated and replaced by those typical of the second and third row of wild-type belts (Fig. 3a, d). The shift in denticle character is accompanied by a reduction in the lateral extension of each abdominal denticle belt so that they become less trapezoidal and more rectangular. This phenotype is very reminiscent of that of *ptc* mutants (compare Fig. 3a and b): in both cases, expansion of the domain of *wg* expression is accompanied by an alteration in the character of the more posterior denticles in each belt. Recent studies have indicated that in normal development, *wg* activity is instrumental in suppressing the formation of denticles^{24,25}; to what extent the ectopic expression of *wg* contributes to the modification of denticle identity seen in HS-*hh* embryos remains to be determined.

Despite the superficial similarity between the HS-*hh* phenotype and that of *ptc* mutant embryos, it is striking that in the latter, a row of denticles characteristic of the first row is frequently duplicated with reversed polarity at the posterior of each denticle belt (Fig. 3b, e). Such duplications correlate well with the ectopic induction of *en* in the middle of each parasegment that is typical of *ptc* mutants^{17,18,22}. It has been suggested that this induction of *en* may result simply from the juxtaposition of *wg*-expressing cells with those competent to respond to *wg* by activating *en* transcription^{8,18}. Yet in HS-*hh* embryos, where the *wg* domain is similarly expanded, no such induction of *en* is observed (Fig. 2). It may be significant that, in contrast to embryos mutant for *ptc*, HS-*hh* embryos carry a functional *ptc* gene that is ectopically expressed in cells adjacent to each expanded *wg* domain. It is possible that this abnormal expression of *ptc* acts to inhibit the ectopic activation of *en*. □

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Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide

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NITRIC oxide, a free-radical gas produced endogenously by several mammalian cell types^{1–6}, has been implicated as a diffusible inter-cellular messenger subserving use-dependent modification of synaptic efficacy in the mature central nervous system^{7–10}. It has been suggested on theoretical grounds that nitric oxide might play an analogous role during the establishment of ordered connections by developing neurons¹¹. We report here that nitric oxide rapidly and reversibly inhibits growth of neurites of rat dorsal root ganglion neurons *in vitro*. In addition, we show that exposure to nitric oxide inhibits thioester-linked long-chain fatty acylation of neuronal proteins, possibly through a direct modification of substrate cysteine thiols. Our results demonstrate a potential role for nitric oxide in the regulation of process outgrowth and remodelling during neuronal development, which may be effected at least in part through modulation of dynamic protein fatty acylation in neuronal growth cones.

Exposure of cultured dorsal root ganglion (DRG) neurons to 3-morpholino-sydononimine (SIN-1), which decomposes spontaneously to release nitric oxide (NO)¹², halts the growth of neurites elongating rapidly on a laminin substratum (Figs 1a, b and 2a, b). Time-lapse video analysis shows that neurite extension ceases within about 2 min after addition of 1 mM SIN-1. Within the next 2–3 min, filopodia and lamellipodia are resorbed as growth cones collapse to a rounded profile, frequently followed by neurite retraction. Calculations based on the rate constants for the two-step hydrolysis of SIN-1 that yields NO¹² and on the half-life of NO (about 10 s under physiological conditions^{1,12}) indicate that the concentration of NO would rise to about 0.75 μM within 1 min and 3.5 μM within 5 min of adding 1 mM SIN-1, reaching a plateau of about 5 μM after 10 min. Growth cone collapse is reversible on removing SIN-1, and repeated cycles of neurite extension and retraction can be elicited by application and withdrawal of SIN-1 (Figs 1b and 2a). Although the interval between removal of SIN-1 and renewed elongation was variable, in most cases a brief exposure to SIN-1 produced a relatively long-lasting inhibition of growth (Fig. 2a, b). Some DRG neurites in our cultures were tipped by motile growth cones but were not actively elongating. For such neurites and for neurites of PC12 cells, which elongate on average much more slowly than DRG neurites, SIN-1 (1–2 mM) inhibited growth cone motility but did not induce retraction (Fig. 1c).

Growth of DRG neurites was not inhibited by solutions containing either 1 mM SIN-1 and 100 μM haemoglobin to bind released NO (ref. 1), or SIN-1 kept at room temperature for 24 h before use, by which time NO is no longer generated but other breakdown products of SIN-1 remain^{12,13} (Fig. 2c). To

test further whether generation of NO is responsible for the observed effects of SIN-1 on growth cones, we treated DRG cultures with a different NO donor, *S*-nitrosocysteine, which decomposes under physiological conditions to yield NO and cystine¹. As with SIN-1, brief exposure to *S*-nitrosocysteine (0.4–1 mM) caused a prolonged, but reversible, inhibition of neurite elongation and growth cone collapse (Fig. 2*d*). Overt neurite retraction following growth cone collapse appeared to be less frequent with *S*-nitrosocysteine than with SIN-1, which could reflect differences in the amount or rate of formation of NO and/or additional by-products. Growth of DRG neurites was not inhibited by solutions originally containing *S*-nitrosocysteine but in which NO production was exhausted (Fig. 2*e*).

Previously, cellular responses to NO have been ascribed to its ability to activate the cGMP-synthesizing haem enzyme, guan-

ylate cyclase, by binding to haem group iron^{1,2}. But growth of DRG neurites was not inhibited by treatment with membrane-permeant cGMP analogues, either 8-bromo-cGMP (1 mM) or dibutyryl cGMP (1 mM) (Fig. 2*f*). Therefore increased production of cGMP is apparently not sufficient to account for the observed responses of growth cones to NO. Exposure to NO under some conditions can also directly modify protein cysteine thiols^{1,14,15}, and we observed previously that exposure of isolated synaptic endings to NO interferes with the post-translational modification of cysteine residues by thioester-linked attachment of palmitate or other long-chain fatty acids (D.T.H. manuscript in preparation). Because it has been suggested that dynamic long-chain fatty acylation of proteins is important for growth cone motility and process outgrowth^{16,31}, we examined the effects of NO on protein fatty acylation in cultured DRG neurons and PC-12 cells under conditions that produced effects on neurite growth.

Figure 3*a, b* shows that incorporation of a radioactive long-chain fatty acid, ³H-palmitate, into a variety of proteins is inhib-

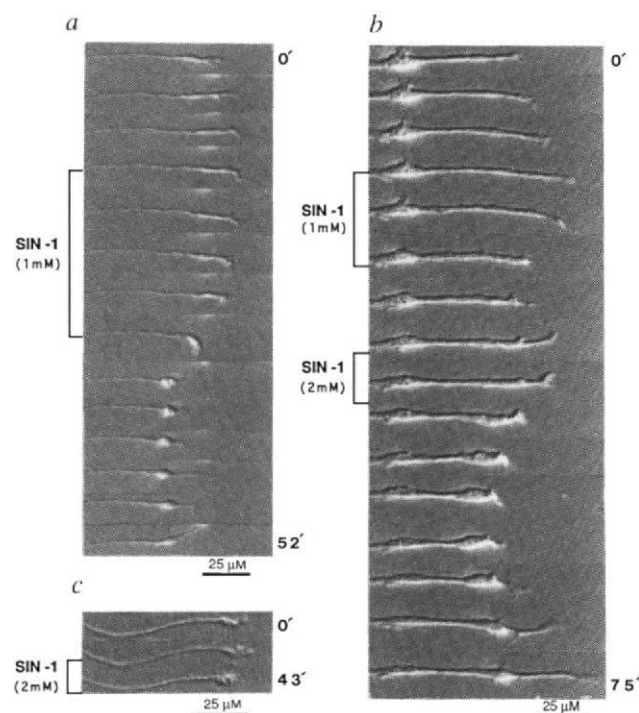


FIG. 1 Inhibition of neuritic growth by nitric oxide. Growing neurites were recorded by video microscopy, and time-series of videomicrographs are shown with elapsed time given in minutes. *a, b*, Exposure of DRG neurons to SIN-1 (1–2 mM) halts extension of elongating neurites, followed by growth cone collapse and retraction, and these effects are reversed by washing with SIN-1-free medium. The sequence depicted in *b* illustrates that repeated cycles of neurite extension and retraction can be elicited by application and withdrawal of SIN-1. *c*, Exposure of PC-12 cells to 2 mM SIN-1 inhibits growth cone motility (as revealed by time-lapse analysis), but does not evoke extensive growth cone collapse or neurite retraction. The inter-frame interval is 4 min in *a*, 5 min in *b*, and 21.5 min in *c*.

METHODS. Two to four days after sciatic nerve crush to stimulate regenerative axon growth²⁶, adult rat DRGs (L4–L6) were removed, dissociated by trituration after treatment with collagenase and trypsin, and plated on laminin-coated dishes²⁷. DRG cultures were maintained in bicarbonate-buffered Ham's F14 medium containing 10% horse serum under 5% carbon dioxide at 37 °C and were used within 24 h of plating. PC-12 cells plated on laminin-coated dishes were maintained in bicarbonate-buffered DMEM containing 5% horse serum and 5% supplemented calf serum under 12% carbon dioxide at 37 °C, and were differentiated with 50 ng ml⁻¹ nerve growth factor (NGF) for at least 5 days before use. Experimental observations were obtained at 37 °C in 25 mM HEPES-buffered medium without serum but containing N1 additives²⁸. SIN-1 was added to cultures as a 200 mM solution in medium, prepared shortly before use and kept on ice. Videomicroscopy employed Hoffmann modulation contrast optics.

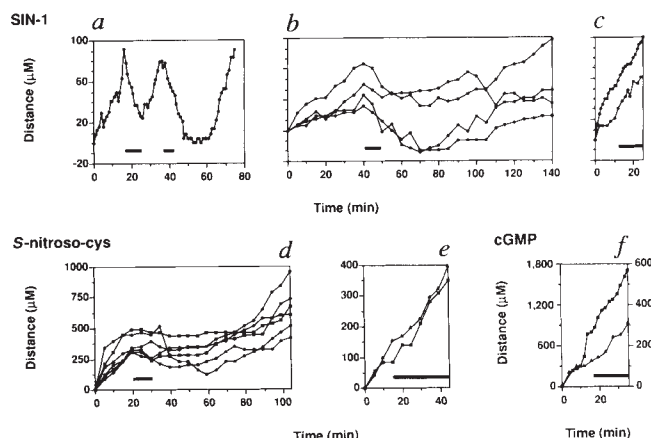


FIG. 2 Graphic representation of the effects of NO and of cGMP analogues on growth of DRG neurites. The position of the leading edge of individual growth cones, measured in single-frame images of video sequences, is plotted as a function of elapsed time. Solid bars indicate the duration of application of test solutions. *a*, Graphic representation of the neurite shown in Fig. 1*b*, illustrating reversible inhibition of growth and retraction induced by sequential treatment with 1 mM and 2 mM SIN-1. Ordinate represents 120 μm in *a–c*. *b*, Results from four additional neurites treated with 1 mM SIN-1. *c*, Maintained growth of two neurites exposed to control media containing either 1 mM SIN-1 and 100 μM haemoglobin (upper curve) or pre-degraded SIN-1 (lower curve). *d*, Inhibition of neurite growth by *S*-nitrosocysteine. Results from six individual neurites are illustrated. *e*, Maintained growth of two neurites exposed to pre-degraded *S*-nitrosocysteine. *f*, Neurite growth is not inhibited by application of either 1 mM dibutyryl cGMP (upper curve, left ordinate) or 1 mM 8-bromo-cGMP (lower curve, right ordinate). Similar results were obtained during continued application for 30 min, and when cGMP derivatives were applied after washout of *S*-nitrosocysteine (not shown).

METHODS. *S*-nitrosocysteine was prepared as a 100 mM stock in 0.5 M HCl according to ref. 29 and stored on ice for up to 1 h. Immediately before use, stock *S*-nitrosocysteine was neutralized by diluting to 2 mM in culture medium containing 5 mM NaOH, and this neutralized stock was added to cell cultures to give a final concentration of 0.4–1 mM *S*-nitrosocysteine. For control observations, unadulterated medium was replaced with medium containing 1 mM SIN-1 plus 100 μM haemoglobin to bind released NO (prepared and filtered immediately before use) or with medium containing 1 mM SIN-1 pre-degraded by storage at room temperature for 24 h before use, or 1 ml of medium originally containing 2 mM *S*-nitrosocysteine was allowed to sit at room temperature for 5 h before addition to 1 ml cultures. The control observations illustrated (*c* and *e*) were obtained in cultures in which reversible inhibition of neurite growth by SIN-1 or *S*-nitrosocysteine was documented before application of control solutions.

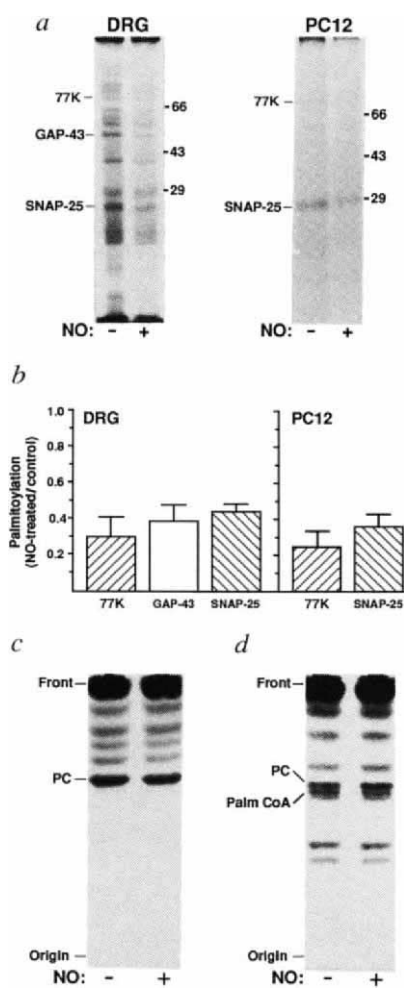


FIG. 3 Nitric oxide inhibits protein long-chain fatty acylation in DRG neurons and PC-12 cells. **a**, **b**, Incorporation of ^3H -palmitate by DRG and PC-12 proteins is reduced by 55–75% in cultures labelled in the presence of 1 mM SIN-1. The positions of GAP-43 and SNAP-25 (see Fig. 4b), and of a 77K substrate present in both DRG neurons and PC-12 cells, are indicated in **a**. GAP-43 expression, which varies among clonal isolates and culture conditions of PC12 cells, was relatively low in the PC12 cells used here and NO effects were not quantified. **c**, **d**, Thin-layer chromatography (TLC) of organic extracts of the DRG cultures analysed in **b** indicates that exposure to NO alters neither overall incorporation of palmitate into cellular lipids (**c**) nor availability of fatty acyl CoA, the acyl donor for protein fatty acylation (**d**). The positions of phosphatidylcholine (PC) and palmitoyl CoA (palm CoA) are indicated. **METHODS.** Cultures were prepared as in Fig. 1. ^3H -palmitic acid (60 Ci mmol $^{-1}$) was added to 1 ml cultures (0.6–1 mCi ml $^{-1}$) in 5–10 μl of DMSO. Labelling was for 30 min (DRG) or 90 min (PC-12) at 37 °C in HEPES-buffered medium containing N1 additives. Labelled cultures were collected by scraping in methanol and extracted with chloroform(5):methanol(5):water(1). Precipitated proteins were solubilized in 0.5% or 1% SDS containing 5 mM DTT, and analysed by SDS-PAGE and fluorography as described¹⁸. For quantification of protein labelling in DRG ($N=4$) and PC-12 ($N=2$) samples, values obtained by scanning densitometry were normalized with respect to labelling of polar lipids, determined by scintillation counting of scraped samples following TLC of total cellular lipids. For TLC analysis, aliquots of organic extracts were either dried down directly to provide samples used to evaluate overall lipid labeling (as in **c**), or re-extracted under neutral conditions to provide samples enriched for palmitoyl CoA³⁰ (as in **d**). Samples were re-dissolved in chloroform:methanol, 2:1, and separated on silica gel plates developed with butanol:acetic acid:water, 5:2:3. Plates were exposed to pre-flashed X-ray film directly (**c**) or after permeation with PPO (**d**). The positions of phosphatidylcholine and palmitoyl CoA were determined with reference to ^3H -labelled standards run in parallel.

ited by 55–75% in both DRG neurons and PC-12 cells exposed to NO. Inhibition of palmitoylation by NO appears to be a direct effect on protein acylation, because NO does not interfere with the uptake of ^3H -palmitate, with incorporation of palmitate into cellular lipids, or with conversion of fatty acid to the acyl donor for protein fatty acylation, palmitoyl-CoA (Fig. 3c, d). The sensitivity of the palmitoyl-protein linkage to hydrolysis by neutral hydroxylamine (Fig. 4a) indicates that DRG and PC-12 proteins incorporate palmitic acid predominantly by way of a thioester linkage to cysteine residues^{16–18}. These results are consistent with inhibition of protein fatty acylation through modification of cysteine thiols on substrate proteins^{1,14}, although regulation by NO of acylating and/or deacylating enzymes is also possible.

Evidence for NO inhibition of growth cone protein palmitoylation was provided by two-dimensional electrophoresis (Fig. 4b), which identified two prominent NO-sensitive substrates as GAP-43 and SNAP-25, previously described as major palmitoylated proteins of axon endings^{16,18–21}. GAP-43 is an abundant growth cone constituent expressed by most developing neurons¹⁹. SNAP-25 is a synaptic protein²⁰ which may play a role in late stages of axon growth and in synaptogenesis²¹. Although our results do not establish a causal link between the effects of NO on protein palmitoylation and its influence on neurite growth, growth cone collapse similar to that induced by NO is evoked by homologues of the antibiotic tunicamycin which selectively inhibit protein long-chain fatty acylation³¹. Thus, inhibition of ongoing palmitoylation of growth cone proteins appears to pro-

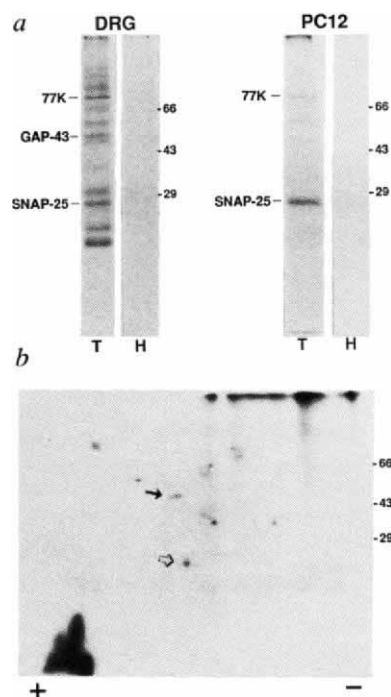


FIG. 4 Thioester-linked long-chain fatty acylation of axon terminal proteins in DRG neurons and PC-12 cells. **a**, ^3H -palmitate is removed from DRG and PC-12 proteins by 1 M hydroxylamine (H) at pH 7 but not by 1 M Tris (T) at pH 7, indicating that labelled fatty acid is linked to cysteine residues through thioester bonds^{16–18}. **b**, High-resolution two-dimensional gel electrophoresis of ^3H -palmitate-labelled DRG proteins identifies GAP-43 (closed arrow; $M_r \sim 50\text{K}$, $pI \sim 4.3$) and SNAP-25 (open arrow; $M_r \sim 25\text{K}$, $pI \sim 4.4$), previously characterized as major palmitoylated proteins of axon endings^{16,18–21}. **METHODS.** Cultures were prepared (see Fig. 1) and labelled with ^3H -palmitate (see Fig. 3). SDS-PAGE gels of labelled proteins were incubated for 4 h at room temperature in 20 vols of 1 M Tris (pH 7) or 1 M hydroxylamine (pH 7) and prepared for fluorography as described¹⁸. Two-dimensional gel electrophoresis was as described¹⁸.

vide one mechanism sufficient to trigger the non-cGMP pathway(s) which mediates the effects of NO on elongating neurites.

Our findings, together with previous evidence of a role for NO in long-term potentiation and depression of synaptic efficacy in the adult central nervous system^{7,10}, support the proposition that NO participates in signalling mechanisms that serve to regulate both synaptic plasticity and axonal growth. NO might play a role in the guidance of elongating axons, as suggested for other molecules that produce growth cone collapse *in vitro*^{22,23}. In addition, because the endogenous production of NO can be regulated by activation of neuronal glutamate receptors^{2,3,24}, the influence of NO on growth cone behaviour may be of particular importance during the activity-dependent remodelling of axon terminals within target structures which subserves the development of ordered patterns of neuronal connectivity^{11,25}. □

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GABA_A receptor needs two homologous domains of the β -subunit for activation by GABA but not by pentobarbital

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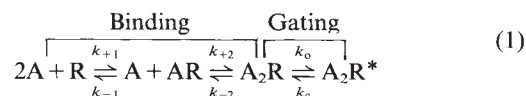
THE predominant inhibitory neurotransmitter of the brain, GABA (γ -aminobutyric acid), activates chloride-selective ion pores integral to the receptor complex. Subunits comprising the presumed hetero-pentameric GABA channel have been cloned^{1–4}, but little information is available on the domains important for activation. Rat wild-type or mutated α_1 -, β_2 - and γ_2 -subunits (designated α , β and γ) were coexpressed in *Xenopus* oocytes and examined electrophysiologically. We report here the identification of two separate and homologous domains of the β -subunit, each of which contributes a tyrosine and threonine essential for activation by GABA. Conservative substitution of each of these four amino acids dramatically decreased GABA channel sensitivity to activation by GABA and the GABA agonist muscimol. These substitutions, however, did not impair activation by the barbiturate pentobarbital, indicating these two different classes of agonists activate GABA channels through distinct mechanisms. We also present evidence suggesting that the two identified domains of the β -subunit contribute a major component of the GABA receptor.

An extensive mutational analysis of the amino-terminal region of the β -subunit (Table 1) has identified four amino acids (tyrosine at position 157 (Y157), T160, T202 and Y205; Fig. 1a) that when conservatively mutated, dramatically impaired the activation of the GABA channel by GABA. Figure 1b shows membrane currents in response to bath application of GABA to oocytes expressing $\alpha\beta\gamma$ (wild type), $\alpha\beta_{Y157F}\gamma$ (tyrosine at position 157 of the β -subunit mutated to phenylalanine), $\alpha\beta_{T160S}\gamma$, $\alpha\beta_{T202S}\gamma$ and $\alpha\beta_{Y205F}\gamma$ subunit combinations. Figure 1c shows

plots of the peak of the currents versus GABA concentration from oocytes expressing wild-type (filled symbols) and mutant (empty symbols) GABA channels. Mutation of either tyrosine produced a comparable decrease in sensitivity to GABA (56- and 51-fold increase in the half-maximal effective concentration (EC_{50}) (Table 1), whereas mutation of either threonine also produced a comparable decrease in sensitivity to GABA (17- and 21-fold). Substitution of β_{Y157} or β_{Y205} with serine or asparagine produced an even greater decrease in GABA sensitivity than substitution with phenylalanine (Table 1), suggesting the aromatic ring may be essential for GABA-mediated activation. Investigations of nicotinic acetylcholine^{5–9} (nACh)- and glycine-operated channels^{10,11} suggest that aromatic rings may be important in ligand-receptor interactions. In addition, β_{T202} of the GABA channel corresponds to a threonine (α_{T204}) implicated in agonist binding to the glycine receptor¹¹.

Excluding the four amino acids described in Fig. 1, mutations of several other residues in this region had little or no apparent effect on GABA sensitivity (Fig. 2a, b and Table 1). The α - and γ -subunits also contain tyrosine residues at positions corresponding to β_{Y157} (α_{Y159} , γ_{Y172}) and β_{Y205} (α_{Y209} , γ_{Y220}) (Fig. 2c). Mutational analysis demonstrated that these corresponding tyrosines in the α - and γ -subunits do not play a role in GABA-mediated activation (Fig. 2d).

Possible mechanisms by which the mutations affected GABA channel sensitivity were considered in terms of a working hypothesis of GABA channel activation. A Hill coefficient greater than one (Table 1) requires the binding of at least two agonist molecules to open the channel, and thus the simplest gating scheme would be:



where A is the agonist, R is the receptor, A_2R is the doubly bound closed GABA channel, and A_2R^* is the doubly bound open GABA channel¹². This gating scheme is an oversimplification^{13,14}, but is a valid model for this discussion.

If a mutation impaired GABA binding in scheme 1, the EC_{50} would increase. Because the effect of the mutation could be overcome by increasing the agonist concentration, the maximum GABA-activated current would not be depressed. Alternatively,

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if a mutation impaired the opening or closing kinetics subsequent to agonist binding (gating), the increase in EC_{50} would be accompanied by a decrease in the maximum which could not be overcome by increasing the agonist concentration. This is shown in Fig. 3a, which plots predicted dose-response relationships assuming scheme 1 (see legend for rate constants) with increasingly impaired gating kinetics. The filled circles in Fig. 3b further demonstrate the dramatic depression in the maxima associated with moderate increases in the EC_{50} predicted by impaired gating kinetics.

To distinguish between effects of the four mutations on agonist binding versus gating, we compared maximum GABA-activated currents in the wild-type and mutant GABA channels. Figure 3b shows the fold increase in EC_{50} versus the fold decrease in the maximum current for the four mutants (filled squares). The lack of depression in the maximum for $\alpha\beta_{Y157F}\gamma$ is consistent with an impairment of agonist binding. Although $\alpha\beta_{T160S}\gamma$, $\alpha\beta_{T202S}\gamma$ and $\alpha\beta_{Y205F}\gamma$ all demonstrated a moderate depression in the maximum, the accompanying large increase in the EC_{50} suggests the effect of these three mutations could not be accounted for by a disruption of channel gating. Because the

functional coupling of agonist binding to channel opening requires a structural coupling between the binding and gating domains, it is tempting to speculate that the three mutations impaired binding, and perhaps to a lesser extent, gating. But we cannot rule out that the depression of the maxima resulted from a disruption of channel assembly. The observed decrease in the Hill coefficient for $\alpha\beta_{T160S}\gamma$, $\alpha\beta_{T202S}\gamma$ and $\alpha\beta_{Y205F}\gamma$ (Table 1) is consistent with either a differential effect on the two GABA-binding steps or a partial effect on gating. Comparisons of ligand binding and single-channel kinetics between wild-type and mutant GABA channels might help resolve the binding/gating issue.

Data presented so far, coupled with photoaffinity labelling studies^{15,16}, implicate a major role for the β -subunit in agonist binding. In support of this, functional GABA channels are not observed in oocytes with coexpression of α - and γ -subunits, whereas coexpression of $\alpha\beta$ and $\beta\gamma$ subunits are functional¹⁷. But domains from neighbouring subunits (α and γ) may contribute to the binding site because an amino acid of the α_1 subunit has been identified that, when mutated (F64L), impairs activation of the GABA channel¹⁸.

The potent GABA agonist muscimol is three times more

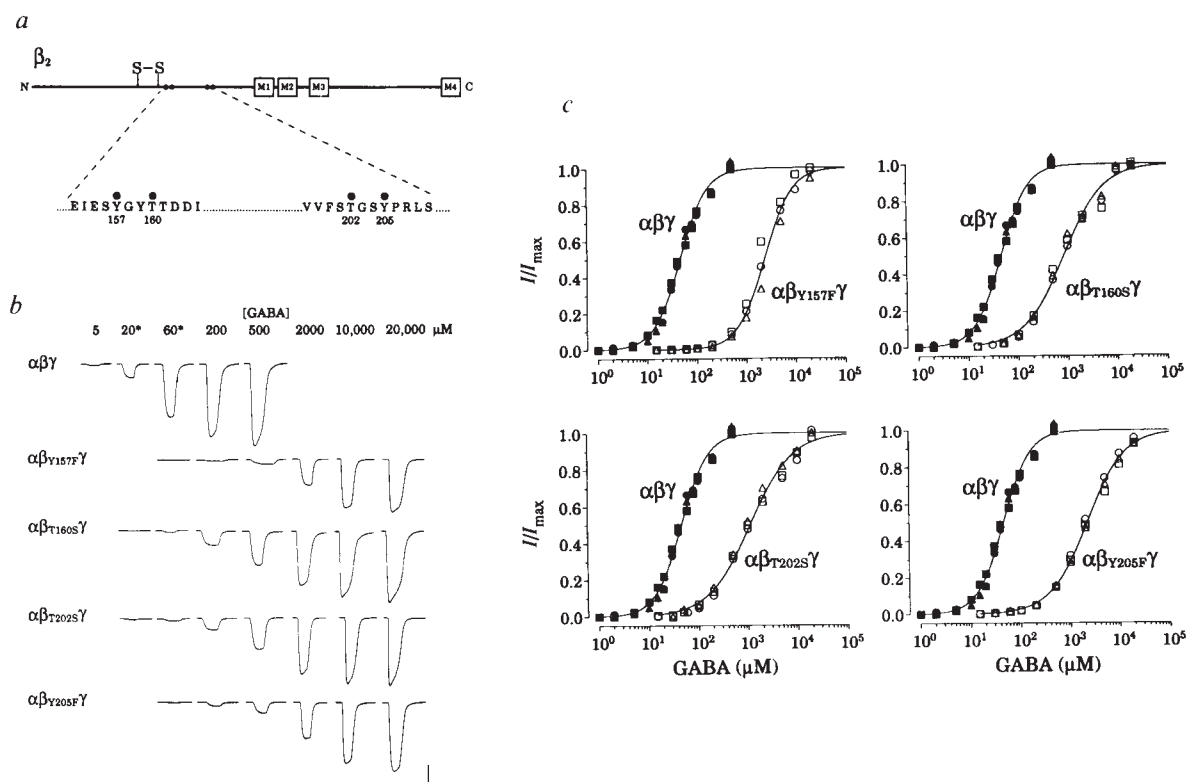


FIG. 1 Identification of four amino acids of the β -subunit (β_{Y157} , β_{T160} , β_{T202} and β_{Y205}) crucial for GABA-dependent gating. a, The four crucial amino acids are on the N-terminal extracellular domain between the disulphide bridge and the first membrane-spanning domain (M1). b, GABA-activated currents from oocytes expressing wild-type or mutant GABA channels. GABA was bath-applied to the oocytes at the concentrations indicated at the top. The asterisks at 20 and 60 μ M indicate that the GABA concentrations for the $\alpha\beta_{T160S}\gamma$ combination were actually 15 and 50 μ M. The calibration bar is 20, 17, 20, 17 and 20 s and 100, 400, 500, 400 and 100 nA for the five rows of traces, respectively. Maximum currents should not be compared as these data represent different amounts of injected cRNA. c, The peak of the GABA-activated currents were divided by the maximum current and plotted versus GABA concentration. In each case, data from three individual oocytes were plotted and in all four plots, the same $\alpha\beta\gamma$ data were replotted for the data. The solid lines are the best fit of the Hill equation to the data.

METHODS. All experiments presented here are coexpression of α -, β -

and γ -subunits because coexpression of α -, β - and γ -isoforms reproduces the main features of native GABA channels²⁶⁻²⁸. cDNAs encoding the α_1 -, β_2 - and γ_2 -subunits were isolated from rat brain RNA using the polymerase chain reaction²⁹ and sequenced until one was found which matched the published amino-acid sequences. Site-directed mutagenesis was achieved with the Altered Sites Kit (Promega, Madison WI). *In-vitro* transcribed cRNA for the desired subunit combinations were mixed in equal ratios, diluted 5- to 30-fold, and injected into the oocytes from a micropipette using positive pressure. In all cases, at least two copies of each clone were tested. Standard two-electrode voltage-clamp techniques (9050A, Knight Indust. Tech., Miami) were used to record currents in response to application of agonist. To quantify the alteration in GABA sensitivity, dose-response relationships were fitted with the Hill equation: $I = I_{max}/1 + (EC_{50}/[A])^n$ where I is the peak current at a given concentration of agonist A, I_{max} is the maximum current, EC_{50} is the concentration of agonist yielding a current half the maximum, and n is the Hill coefficient.

TABLE 1 Parameters determined from fitting the Hill equation to GABA, muscimol, and pentobarbital dose-response relationships

Subunit combination	EC ₅₀ (μM)	Hill coefficient*	Number of oocytes tested
GABA-mediated activation			
Wild type			
αβγ	45.8 ± 3.6	1.57 ± 0.09	5
β-subunit mutants			
αβ _{Y143F} γ†	17.2 ± 3.6	1.72 ± 0.38	3
αβ _{T151A} γ†	173.1 ± 45.1	1.36 ± 0.17	4
αβ _{S156T} γ†	77.9 ± 12.6	1.19 ± 0.01	2
αβ _{Y157F} γ†	2,570 ± 646	1.50 ± 0.20	4
αβ _{Y157S} γ	43,580 ± 6,800	1.46 ± 0.10	3
αβ _{Y157N} γ	No current detected up to 20,000 μM‡		9
αβ _{Y159F} γ	228.7 ± 27.0	1.28 ± 0.21	3
αβ _{Y159S} γ†	149.5 ± 27.8	1.17 ± 0.03	4
αβ _{T160S} γ†	761.5 ± 163.3	1.14 ± 0.10	4
αβ _{T160A} γ§	379.3 ± 79.8	1.28 ± 0.15	4
αβ _{T161A} γ†	120.4 ± 44.4	1.30 ± 0.22	3
αβ _{YF166FY} γ†	32.9 ± 3.6	1.64 ± 0.33	3
αβ _{T176A} γ†	61.1 ± 11.2	1.33 ± 0.04	4
αβ _{T179A} γ†	37.1 ± 11.7	1.41 ± 0.15	4
αβ _{Y191F} γ†	57.7 ± 8.5	1.33 ± 0.10	3
αβ _{S201T} γ†	58.6 ± 9.0	1.41 ± 0.14	2
αβ _{S201A} γ	77.9 ± 12.6	1.30 ± 0.06	4
αβ _{T202S} γ†	948.2 ± 203	1.13 ± 0.15	5
αβ _{T202A} γ	No current detected up to 20,000 μM‡		5
αβ _{S204A} γ†	44.9 ± 10.1	1.28 ± 0.13	3
αβ _{Y205F} γ†	2,323 ± 586	1.17 ± 0.13	7
αβ _{Y205S} γ	No current detected up to 20,000 μM‡		6
αβ _{Y205N} γ	No current detected up to 20,000 μM‡		9
αβ _{FY220VF} γ†	20.0 ± 1.4	1.39 ± 0.02	2
α-subunit mutants			
α _{Y159S} βγ	44.9 ± 4.5	1.62 ± 0.18	4
α _{Y161S} βγ	47.3 ± 6.1	1.67 ± 0.06	3
α _{Y209F} βγ	34.9 ± 4.5	1.38 ± 0.04	3
α _{Y209S} βγ	38.2 ± 12.2	1.38 ± 0.10	4
γ-subunit mutants			
αβγ _{Y172S}	40.4 ± 5.0	1.49 ± 0.14	4
αβγ _{Y174S}	90.2 ± 28.8	1.09 ± 0.19	4
αβγ _{Y220S}	38.4 ± 6.2	1.43 ± 0.10	2
Muscimol-mediated activation			
αβγ	14.3 ± 3.2	1.42 ± 0.09	4
αβ _{Y157F} γ	2,570 ± 467	1.25 ± 0.09	3
αβ _{T160S} γ	145.7 ± 33.1	1.59 ± 0.20	3
αβ _{T202S} γ	536.3 ± 169	1.21 ± 0.17	3
αβ _{Y205F} γ	296.6 ± 78.9	1.37 ± 0.35	3
Pentobarbital-mediated activation			
αβγ	636.3 ± 53.7	3.78 ± 0.66	3
αβ _{Y157F} γ	616.6 ± 13.4	3.82 ± 0.03	2
αβ _{Y157S} γ	788.9 ± 62.3	2.87 ± 0.14	3
αβ _{T160S} γ	379.0 ± 84.0	3.26 ± 0.37	4
αβ _{T160A} γ	413.0 ± 68.6	3.79 ± 0.37	6
αβ _{T202S} γ	559.7 ± 25.1	2.23 ± 0.13	3
αβ _{T202A} γ	439.5 ± 31.8	2.82 ± 0.14	2
αβ _{Y205F} γ	552.2 ± 0.7	4.24 ± 0.42	2
αβ _{Y205S} γ	464.2 ± 30.9	2.41 ± 0.10	3

The EC₅₀ (concentration of GABA required for half-maximal activation) and Hill coefficient are the mean ± s.d. The primary mutants discussed in the text are shown in bold.

* Some caution should be exercised in interpreting the Hill coefficient as this parameter is influenced by characteristics of both agonist binding and ion-channel gating.

† Mutants plotted in Fig. 2b.

‡ These combinations were expressed because pentobarbital-activated currents were observed. When excessive amounts of cRNA were injected, small GABA-activated currents were detected at concentrations of GABA > 10,000 μM.

§ This combination produced a ~500-fold decrease in the maximum GABA-activated current, suggesting an effect on assembly or gating.

efficacious in activating the wild-type GABA channel than GABA (Table 1). Figure 3c compares the fold increase in EC₅₀ for GABA (empty bars) and muscimol (striped bars) with mutation of the four amino acids in the two domains. All four mutations increased the EC₅₀ for muscimol, although the relative shifts in sensitivity were different for GABA and muscimol, presumably reflecting their different structures. By far the largest decrease in muscimol sensitivity was observed for the αβ_{Y157F}γ combination with an EC₅₀ that was 180-fold greater than the wild-type EC₅₀ for muscimol. This increase in the muscimol EC₅₀ is about threefold greater than the increase in the GABA EC₅₀

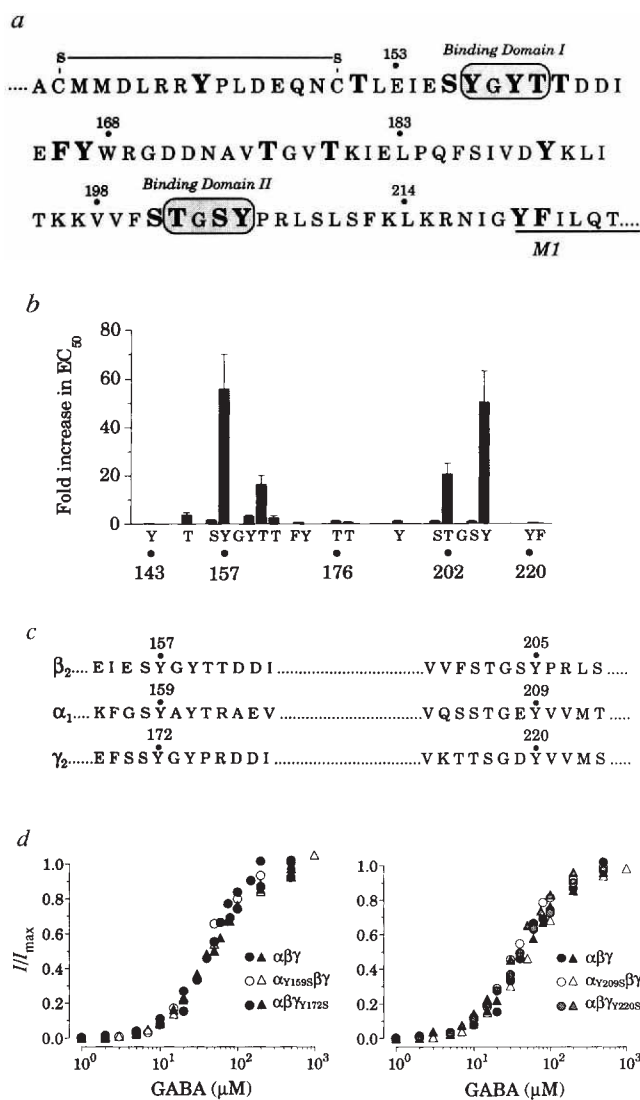


FIG. 2 Two separate domains of the β-subunit are required for GABA-dependent activation. **a**, The amino-acid sequence of the β-subunit flanking and intervening the four crucial amino acids is shown. All amino acids that have been mutated and coexpressed with α- and γ-subunits are indicated in large bold letters. The two binding domains are inverted repeats with the consensus sequence being a glycine adjacent to a variant residue flanked on either side by the tyrosine and threonine. **b**, Fold increase in EC₅₀ for β-subunit mutants indicated by asterisks in Table 1. These data identify two crucial domains (boxed in **a**) around positions 157 and 202 of the β-subunit. The error bars are the standard deviation. **c**, Alignment of the amino-acid sequences flanking the two domains of the β-, α- and γ-subunits. The crucial tyrosines are conserved across all three subunits. **d**, The tyrosines in the α- and γ-subunits homologous to β_{Y157} and β_{Y205} were mutated to serine and coexpressed with their wild-type counterparts. In both plots, the wild-type data is replotted for comparison.

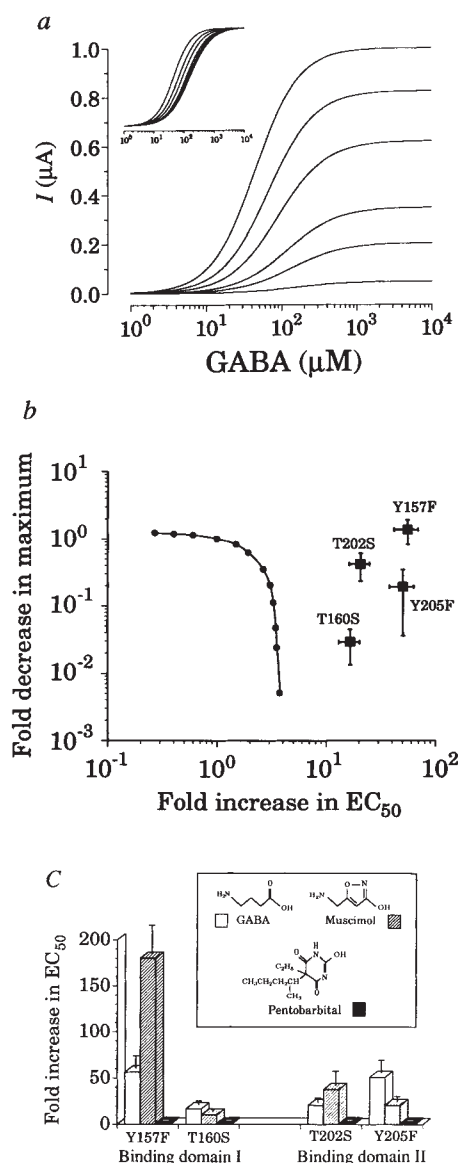


FIG. 3 Decrease in agonist sensitivity of the mutants is consistent with an effect on agonist binding. **a**, Predicted dose-response relationships for scheme 1 assuming the mutation impaired the gating kinetics subsequent to agonist binding. The rate constants used to model the wild type GABA channel were (in s^{-1}): k_o , 510; k_c , 2,100; k_{-2} , 260; k_{+2} , $2 \mu M^{-1}$; k_{-1} , 130; k_{+1} , $4 \mu M^{-1}$. Scheme 1, with the above rates, yielded an EC_{50} of 44.6 μM and a Hill coefficient of 1.49. The gating impairment was modelled as a decrease in the opening rate constant (transition from A_2R to A_2R^* in Scheme 1). The inset is a plot of the same data, but normalized to the maximum to help comparison of the EC_{50} s. **b**, The filled circles plot the fold increase in EC_{50} versus the fold decrease in the maximum for the predicted data in **a** with the opening rate varied from 20,000 to $2 s^{-1}$. The filled squares plot the fold increase in the EC_{50} versus the fold decrease in the maximum for the four mutants described in Fig. 1. **c**, Comparison of EC_{50} shifts for activation of the mutant GABA channels by GABA, muscimol and pentobarbital.

METHODS. The opening and closing rate constants (k_o and k_c) for scheme 1 were determined from a preliminary single-channel kinetic analysis of wild-type GABA channels expressed in oocytes (data not shown). Briefly, open and shut interval durations were determined from clusters of single-channel activity in the presence of 25 μM GABA. k_o and k_c were then determined by fitting a simple kinetic scheme (similar to scheme 1) to open and shut dwell-time distributions using an iterative Q-matrix method³⁰. Values for k_{+1} , k_{+2} , k_{-1} and k_{-2} were based on an assumption of equivalent binding sites. The analysis presented in this figure requires a channel with a relatively low efficacy^{31,32} (such as, $k_o/k_c < 10-20$). The low value we observed for this ratio (~ 4) agrees with what others have found^{13,33,34}. For measurements of the maxima, the amount of cRNA was judged and matched by interpolation of lanes (1% formaldehyde gel) containing different dilutions of the corresponding cRNA. Injections of known amounts of ^{35}S -ATP into oocytes demonstrated that the injected volumes were nearly equal. The maximum current was then determined by applying a concentration of GABA > 10 times the EC_{50} : $\alpha\beta\gamma$, 500 μM ; $\alpha\beta_{T160S}\gamma$ and $\alpha\beta_{Y205S}\gamma$, 10,000 μM , $\alpha\beta_{Y157F}\gamma$ and $\alpha\beta_{Y205F}\gamma$, 20,000 μM .

observed with the same mutation. Thus, β_{Y157} may account for the ~ 3 -fold greater sensitivity of the wild-type GABA channel to muscimol as compared to GABA. Because the major structural difference between GABA and muscimol is the isoxazole ring (Fig. 3c), β_{Y157} may interact with muscimol at the 'carboxy-like' 3-isoxazolol moiety.

The barbiturate pentobarbital modulates, as well as directly activates, GABA channels¹⁹⁻²¹. The direct activation is thought to underlie, in part, pentobarbital's anaesthetic actions on the central nervous system²². Competitive antagonists at the GABA receptor also antagonize pentobarbital-dependent activation suggesting these two agonists share receptor domains²¹. In contrast to GABA and muscimol, sensitivity to activation by pentobarbital was not impaired in any of the four mutants (Table 1, and filled bars in Fig. 3c). Even $\alpha\beta_{T202A}\gamma$ and $\alpha\beta_{Y205S}\gamma$, which were unresponsive to GABA, did not demonstrate an impairment in pentobarbital-mediated activation (Table 1). These data provide direct evidence that GABA and pentobarbital activate GABA channels through distinct mechanisms. One possibility is pentobarbital may activate the GABA channel through hydrophobic interactions. If true, the pentobarbital binding site may prove more difficult to identify using site-directed mutagenesis.

The data presented in this study are consistent with two separate GABA-binding domains (BD I and BD II) on the β -subunit,

each of which contribute a tyrosine and a threonine crucial for activation by GABA (Fig. 2a). The BD I and BD II sequences are homologous (inverted repeats), with the consensus sequence being a glycine adjacent to a variant residue (tyrosine and serine in BD I and BD II, respectively) flanked on either side by the crucial threonine and tyrosine. The conserved glycines might be required to make hairpin turns and put the tyrosines and threonines in proper register for interaction with the agonist. Thus, the GABA-binding site, as in other ligand-binding sites of proteins^{23,24}, may consist of multiple loops. The first and fourth amino acids in a hairpin turn (which would be the crucial tyrosine and threonine) typically form hydrogen bonds between the main chain amino and carboxy groups^{25,25}. Although we have suggested the tyrosine and threonine in each domain both interact with the agonist, it is also possible that mutation of the threonine could disrupt the loop structure, thereby shifting the position of the essential tyrosine. Thus, the threonine substitution might affect GABA sensitivity indirectly. Regarding the more specific role of the two domains in the presumed two GABA-binding sites per functional channel, BD I and BD II may interact with a common GABA molecule and be part of the same binding site, or BD I and BD II may interact independently with two GABA molecules and thus contribute separately to the two binding sites. Verification of such structural pre-

dictions must await the crystallization and structural resolution of these membrane proteins. □

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The probability of transmitter release at a mammalian central synapse

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WHEN an action potential reaches a synaptic terminal, fusion of a transmitter-containing vesicle with the presynaptic membrane occurs with a probability (p_r) of less than one¹. Despite the fundamental importance of this parameter, p_r has not been directly measured in the central nervous system. Here we describe a novel approach to determine p_r , monitoring the decrement of NMDA (*N*-methyl-D-aspartate)-receptor mediated synaptic currents in the presence of the use-dependent channel blocker MK-801 (ref. 2). On a single postsynaptic CA1 hippocampal slice neuron, two classes of synapses with a sixfold difference in p_r are resolved. Synapses with low p_r contribute to over half of transmission and are more sensitive to drugs enhancing transmitter release. Switching between these two classes of synapses provides the potential for large changes in synaptic efficacy and could underlie forms of activity-dependent plasticity.

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To determine the probability of release (p_r) at a central glutamatergic synapse, we monitored the decrement of elicited NMDA-mediated transmission as a function of repeated trials in the presence of MK-801. Because MK-801 is an irreversible NMDA-channel open channel blocker³, only those receptors on synapses that release transmitter can be blocked, preventing their participation in subsequent transmission. Intuitively, if p_r is high, a large fraction of available synapses will be eliminated with each trial, and transmission will decay quickly. If p_r is low, then a small fraction of synapses will be eliminated with each trial, and transmission will decay slowly.

The relation between the amplitude of NMDA-mediated e.p.s.cs for consecutive trials in the presence of MK-801 is given by

$$R(t+1) = R(t) \times (1-F) \quad (1)$$

where $R(t)$ is the synaptic response for the t th trial; and F , the fraction of NMDA channels blocked by MK-801 in one trial out of all available NMDA channels, is calculated from

$$F = p_r \times p_f \times FB \quad (2)$$

where, FB = fraction of NMDA channels blocked by MK-801 at a synapse that releases transmitter; p_r = probability that a terminal receiving an action potential will release transmitter; p_f = the probability that a fibre stimulated with an extracellular electrode fires an action potential that is conducted to a synaptic terminal.

In MK-801, the predicted decrement in transmission is given by solving difference equation (1):

$$R(t) = R_0 \exp(-t/\tau)$$

where $\tau = -1/\ln(1-F)$ and R_0 is the initial response in MK-801. Thus, from equation (2)

$$p_r = \frac{1 - \exp(-1/\tau)}{p_f \times FB} \approx \frac{1}{\tau \times p_f \times FB} \text{ if } \tau > 5 \quad (3)$$

Therefore if τ , p_f and FB are measured, then the probability of transmitter release, p_r , can be determined.

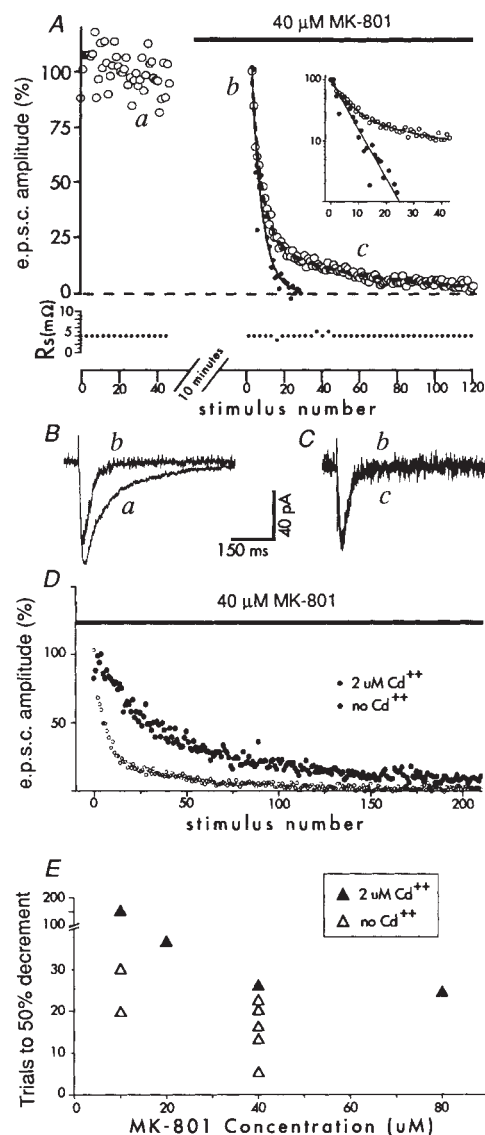
Stable NMDA-mediated synaptic responses between Schaffer collateral/commissural fibres and CA1 neurons were recorded in hippocampal slices in the presence of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)-receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Fig. 1). In MK-801, individual NMDA-mediated e.p.s.cs decayed more rapidly with little effect on the initial slope of the response (Fig. 1B; ref. 4), confirming the open-channel blocking nature of the drug. With continued stimulation, the peak amplitude progressively diminished (Fig. 1A) without changes in response kinetics (Fig. 1C, b, c). This argues against an increase in MK-801 concentration at the synapse after the initial response in the drug and against poor voltage control of NMDA-mediated currents at the subsynaptic membrane. Halting stimulation for 10–30 min after partial or total inhibition of transmission by MK-801 yielded no recovery of the response ($n=3$; data not shown), supporting the irreversibility of channel block.

Equation (3) predicts that p_r is inversely related to τ . We tested this directly by examining the effect of extracellular Cd^{2+} (which blocks presynaptic calcium channels) on the decrement of transmission produced by MK-801 (Fig. 1D). In MK-801, transmission decayed more slowly in the presence of Cd^{2+} ($n=4$) than in its absence ($n=9$; Fig. 1D, E).

To estimate p_r , we used the voltage-dependent Na^+ channel open-channel blocker QX-314 (ref. 5). Extracellular application of this drug produced a use-dependent slowing of action potential conduction ($n=5$, Fig. 2A). To estimate p_r of a population of fibres, antidromic responses were recorded simultaneously from a single CA3 cell and from the surrounding population (Fig. 2B). With repeated stimuli, the same fractional change in conduction velocity was noted in the population as in the individual cell that fired every trial (Fig. 2B, C; $n=3$). This indicates

FIG. 1 Decay of NMDA-mediated responses in the presence of the open-channel blocker MK-801. **A**, top, Normalized peak amplitude of synaptic NMDA currents (empty circles) measured at -10 mV plotted versus stimulus trials. MK-801 ($40 \mu\text{M}$) was applied where indicated followed by interruption of stimuli for 10 min. In parallel experiments without MK-801, the stability of transmission was affected little by a 10 min interruption of afferent stimulation ($N = 7$). Least-square fit (dashed line) of the decrement of transmission in MK-801 to the sum of 2 exponentials gives time constants $\tau_1 = 5.3$ trials (79%) and $\tau_2 = 59$ trials (21%). Normalized peak amplitude of responses (filled circles) to brief (2 ms) application of glutamate (1 mM) in the presence of MK-801 ($40 \mu\text{M}$) and CNQX ($4 \mu\text{M}$) from a different cell overlaid for comparison. Response decrement was fitted well by a single exponential (solid line, $\tau = 5.0$ trials). Inset, Expanded view of response decrement plotted on a logarithmic scale. Bottom, Series resistance plotted versus stimulus number. **B**, Ensemble averages of 30 (**a**) and 2 (**b**) consecutive e.p.s.c.s recorded before and after MK-801 application (epochs indicated in **A**). **C**, Ensemble averages of 2 (**b**) and 30 (**c**) consecutive e.p.s.c.s obtained where indicated in **A** scaled to match peak amplitude. Note close kinetic agreement of normalized traces. Experiments in which the later e.p.s.c.s decayed faster than early e.p.s.c.s were not further analysed. **D**, Peak amplitude of synaptic NMDA currents plotted versus stimulus trial for an experiment with no Cd^{2+} (empty circles) and $2 \mu\text{M}$ Cd^{2+} (filled circles) included in superfusate. Cd^{2+} inhibited transmission by 66% (not shown). $40 \mu\text{M}$ MK-801 produced a slower decrement of transmission in experiment with Cd^{2+} , with greater effect on the initial fast decrement. **E**, Plot of trials required for peak amplitude of NMDA mediated e.p.s.c. to decay to 50% of initial value in MK-801 versus concentration of MK-801 for experiments in $2 \mu\text{M}$ cadmium (filled triangles) and no cadmium (empty triangles).

METHODS. Transverse hippocampal slices were prepared from 11–19-day-old rats and whole-cell recordings were obtained as previously described¹². NMDA currents were isolated by addition of 3 – $10 \mu\text{M}$ CNQX (RBI) and $100 \mu\text{M}$ picrotoxin (Sigma) to the bath. Transmission was elicited (0.1 Hz) with a stimulating electrode placed in the CA1 stratum radiatum. Responses to agonist were elicited by injecting (Picospritzer, General Valve; 2 ms, 20 p.s.i.) glutamate (1 mM) through a patch pipette placed in stratum radiatum. Input (100 – $300 \text{ M}\Omega$) and series (3.5 – $10 \text{ M}\Omega$) resistances were monitored throughout an experiment (**A**). If series resistance deviated more than 30% during an experiment, the experiment was removed from analysis. The decrement of transmission as a function of trials (t) after MK-801 (RBI) application was fit (least-squares, MicroCal Origin) to the function $y(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + y_0$ allowing A_1 , A_2 , τ_1 and τ_2 to vary.



that fibres contributing to the population response also fired with $p_f \sim 1$.

To obtain a simple and direct measurement of *FB*, we monitored the decrement of NMDA-mediated responses to pressure-applied glutamate in the presence of MK-801 (Fig. 1A). In $40 \mu\text{M}$ MK-801 the decrement was fitted well by a monoexponential function with time constant $\tau = 4.0 \pm 0.8$ ($n = 6$), which

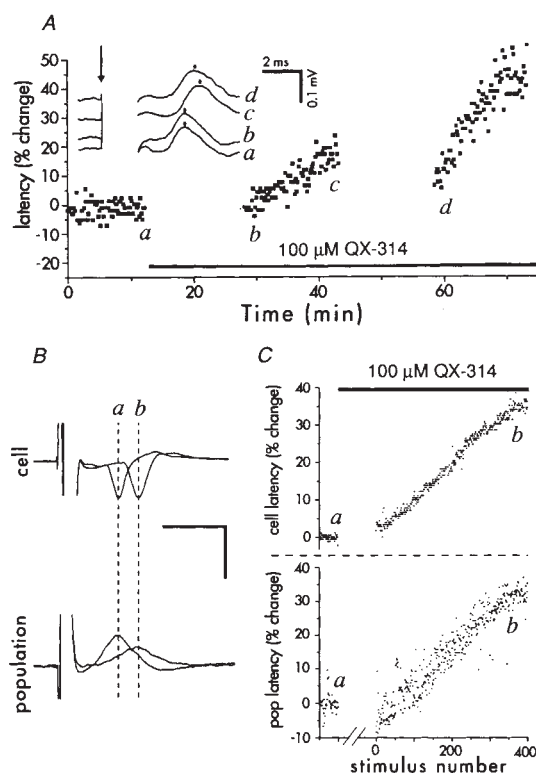
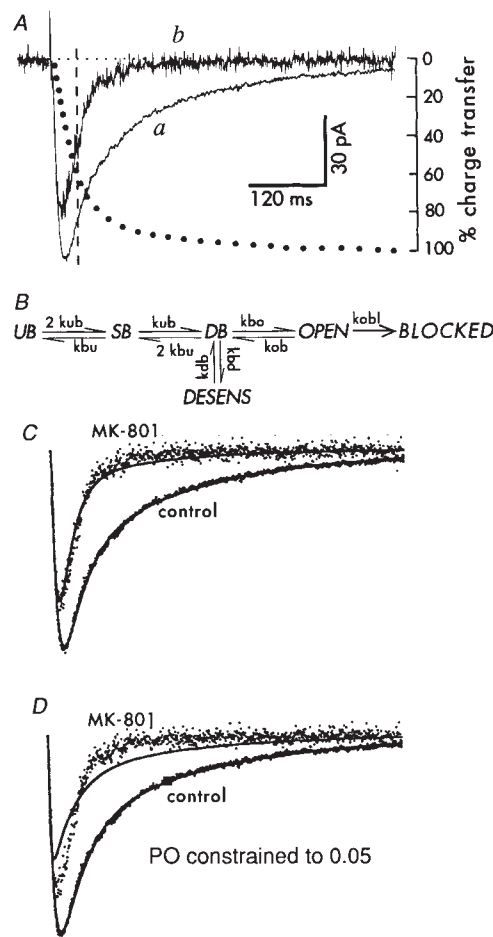


FIG. 2 Fibres excited with an extracellular stimulating electrode fire with high probability. **A**, Plot of change in conduction time versus time for an antidromically activated population of CA3 neurons. QX-314 (RBI) is applied to bath where indicated. Note no increase in conduction time in the absence of stimuli demonstrating a use-dependent block. Inset, Average of 5 sweeps obtained at times indicated on plot. Arrow indicates stimulus artefact; asterisks indicate peak of antidromic response. **B**, Antidromic responses from a single CA3 cell (cell-attached patch, top) and a nearby population of CA3 cells (field electrode, bottom) obtained at time indicated on graph in **C**. Calibration bars: top 4 ms, 20 pA; bottom 4.75 ms, 1.25 mV. Note similar change in relative conduction time in the individual cell as in the population of cells. **C**, Plot of conduction time measured in a single fibre (top) and in the population of cells (bottom). Drug is applied where indicated. The change in conduction time in the single fibre versus that of the population correlate (least squares linear regression) with slope of 1 indicating that the population of cells is affected by QX-314 as much as a single fibre that fires every time. Perfusate contained $10 \mu\text{M}$ CNQX, $80 \mu\text{M}$ MK-801 and $10 \mu\text{M}$ 2-chloro-adenosine (Sigma) to block synaptic transmission.

FIG. 3 Fraction of NMDA receptors blocked by MK-801 during an e.p.s.c. (FB) calculated with two methods. A, e.p.s.cs recorded before (a, average of 30 traces) and during (b, average of the first 2 traces) application of 40 μM MK-801. In the absence of MK-801, e.p.s.cs (10%–90% rise time = 12.4 ± 1.6 ms; biexponential decay with $\tau_1 = 55.7 \pm 3.2$ and $\tau_2 = 225 \pm 16$; $N = 13$) resembled currents resulting from application of saturating concentrations of glutamate to membrane patches^{8,13,14}. Percentage of total charge (integrated current) transferred during synaptic response in MK-801 (filled circles). Time for 60% of charge to be transferred ($t_{60\%} = 41$ ms) indicated by dashed line. In membrane patch studies³ a similar $t_{60\%}$ is observed if [MK-801] = 3–10 μM and 40–65% of patch channels are blocked. The lipophilicity of MK-801 may explain the apparent 4–10-fold difference between drug concentration in the bath and that inferred to be at the synapse. Records are from same experiment as in Fig. 1B. B, Reaction scheme used to model opening and blocking of synaptic NMDA receptors¹⁰. C, e.p.s.c. averages (dots) in the absence and presence of MK-801, with least-squared fits (solid line) obtained using reaction scheme in B. D, e.p.s.c. averages (dots) overlaid with least-square fits (solid line) with $P_{\text{open}} = k_{\text{bo}}/(k_{\text{ob}} + k_{\text{bo}})$ constrained to 0.05. This value for P_{open} , suggested by other studies^{2,15}, is lower than the value from our unconstrained simulations ($P_{\text{open}} = 0.31 \pm 0.05$; $N = 13$). Note the poor fit to e.p.s.c. in MK-801 with such constraints. In addition a 300% increase in receptor number was required (experiment N19T02). A heterogeneity in FB sufficient to account for τ_2/τ_1 (see text) is inconsistent with several lines of evidence. First, the time course of synaptic responses (as can be measured by $t_{60\%}$) was similar for all trials after application of MK-801 (Fig. 1C). For different concentrations of MK-801, $t_{60\%} = 1.6 \times \text{FB} + \text{intercept}$ ($n = 13$). Thus, a sixfold difference in blocking rate (necessary to explain τ_2/τ_1) would show up as a 10-fold increase in $t_{60\%}$ which is not seen. Second, differential binding of agonist (such as non-homogeneous exposure of agonist to channels or homogenous exposure to channels with different agonist affinities) is not consistent with the data obtained with Cd^{2+} . Addition of extracellular Cd^{2+} primarily slowed the faster decrement, τ_1 ($\tau_2/\tau_1 = 2.7 \pm 1.1$ in Cd^{2+} , $n = 4$; $\tau_2/\tau_1 = 5.8 \pm 0.9$ in no Cd^{2+} , $n = 9$; $P < 0.05$; Fig. 1D). If τ_2 corresponds to the population of receptors with lower binding efficacy one would predict a similar or greater effect of Cd^{2+} on this component of transmission. And finally, the monoexponential decrement of responses to exogenously applied glutamate argues against heterogeneity of receptor binding, opening or blocking rates, although admittedly, the temporal and spatial agonist concentration profile or receptors recruited with such application may not faithfully reflect synaptic transmission. We note that if a single vesicle does not saturate all NMDA receptors, our estimate of p_r may be low.

METHODS. In simulations, a brief pulse of transmitter was delivered and model parameters were adjusted to fit optimally synaptic currents recorded with no MK-801 using a least-squares fitting procedure in the SCoFit program (Simulation Resources). With these parameters fixed, blocking rate and receptor number were allowed to vary to fit optimally e.p.s.c. in MK-801. With such a procedure, changes in receptor number



(reflecting variations in transmission during the 10 min interruption) were minimal ($-7 \pm 5\%$; $N = 13$). Glutamate binding rate was constrained to a previously estimated value ($5 \mu\text{M}^{-1} \text{s}^{-1}$ ref. 14), and blocking rate, k_{obl} , was set to 0 in fits to currents recorded in no MK-801. State and rate constant abbreviations along with values obtained in no MK-801 ($N = 13$): UB, unbound; SB, single bound; DB, double bound; DESENS, desensitized; k_{ub} , binding rate; k_{bu} , unbinding rate (9.5 ± 0.6); k_{bo} , opening rate (25 ± 1.5); k_{ob} , closing rate (59 ± 5); k_{bd} , desensitization rate (16 ± 1.7); k_{db} , resensitization rate (13 ± 1.2); k_{obl} , block rate. Units of k_{ub} are $\mu\text{M}^{-1} \text{s}^{-1}$ and of all other rates are s^{-1} .

implies (from equation (3)) $\text{FB} = 25 \pm 6\%$. But spatial-temporal agonist concentration profiles or the receptor pools activated may differ for synaptic and exogenously applied transmitter. Thus, two other independent methods were used to determine FB. One method uses the observation that the fraction of NMDA channels on isolated membrane patches blocked by MK-801 during a single brief application of glutamate is related to the time required for 60% of the charge to be transferred, $t_{60\%}$ (ref. 3; Fig. 3 legend). In hippocampal slices with 40 μM MK-801, $t_{60\%} = 65 \pm 8$ ms ($n = 6$, Fig. 3A) implying (from ref. 3) that about 40–50% of channels at synapses that release transmitter are blocked (Fig. 3 legend). For a third independent estimate of FB, we used a kinetic model that accounts for the macroscopic behaviour of NMDA currents from isolated patches and from synapses of cultured neurons (Fig. 3B, refs 6–8). Least-squares fits of this model to experimental e.p.s.cs in the absence and presence of 40 μM MK-801 (Fig. 3C) yielded a value of $\text{FB} = 30 \pm 8\%$ ($n = 6$), generally in agreement with the values for FB obtained above.

To calculate τ , the decrement of peak e.p.s.c. amplitude produced by MK-801 was fitted to exponential functions (Fig. 1 legend). In general (8 of 9 experiments), the decrement over

trials (t) could be best fitted by the sum of two exponentials, $A_1 \exp(t/\tau_1) + A_2 \exp(t/\tau_2)$ with $A_2/(A_1 + A_2) = 0.54 \pm 0.05$ and $\tau_2/\tau_1 = 6.4 \pm 0.7$ ($N = 8$; Fig. 1A). This biexponential decrement indicates heterogeneity in p_r , FB and/or p_r . To account for the observed $A_2/(A_1 + A_2)$ and τ_2/τ_1 with multiple values of p_r , approximately 85% of axons must have a $p_r < 0.15$, which is inconsistent with the results in Fig. 2. A heterogeneity of FB would require receptors that are differentially blocked by MK-801, those with greater FB being removed from transmission earlier. Such heterogeneity is not supported by (1) the similar response kinetics throughout the block (Fig. 1C and Fig. 3 legend); (2) the observation that Cd^{2+} differentially affects the two components of the biexponential decrement (Fig. 1D and Fig. 3 legend); or (3) the monoexponential decrement of responses to brief exogenous application of glutamate in the presence of MK-801. Thus our data argue against heterogeneity in p_r or FB, suggesting that there are two populations of synapses with high and low p_r . For each of the eight experiments showing a biexponential decrement of transmission in MK-801, τ_1 and τ_2 were computed together with FB (using the kinetic model) allowing calculation of high $p_r = 0.37 \pm 0.04$ and low $p_r = 0.06 \pm 0.01$.

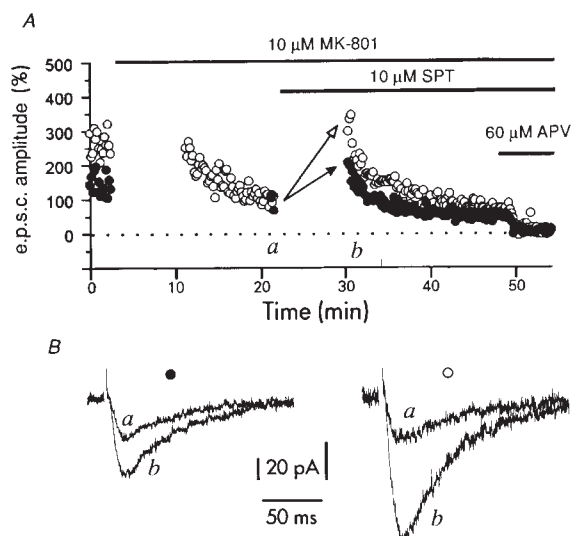


FIG. 4 Synapses with low probability of release are more sensitive to drugs enhancing release. A, Peak NMDA synaptic currents in 4 μ M CNQX plotted versus time for two inputs onto a single neuron, normalized to current amplitude at time *a*. After addition of MK-801, one pathway (empty circles) was stimulated 60 times (0.1 Hz), the other pathway (filled circles) was stimulated 3 times (0.1 Hz). Addition of SPT (10 μ M, RBI) to disinhibit transmission by blocking presynaptic adenosine receptors, produces a larger enhancement in the pathway retaining primarily low p_r synapses (empty circles). B, e.p.s.c. averages obtained where indicated on graph from two inputs (empty and filled circles).

To test further for synapses with differing p_r , we designed an experiment to isolate these two populations and examined the effect of drugs that act presynaptically. We reasoned that synapses with a lower p_r should be affected more by manipulations that increase release^{9–11}. Synapses with a low p_r were partially isolated by stimulating afferent inputs repeatedly (more than 40 times at 0.1 Hz) in the presence of MK-801 (Fig. 4A). This would be expected to remove predominantly synapses with a higher p_r . Another input to the cell, stimulated for only a few trials in MK-801, would be expected to retain both high and low p_r synapses. Bath application of agents acting presynaptically produced a greater enhancement in the pathway that had been repeatedly stimulated in MK-801 (Fig. 4A, B). The enhancement was $37 \pm 4\%$ greater with 8-(*p*-sulphophenyl)theophylline (10–20 μ M, $n=4$, $P<0.05$, paired *t*-test), and $90 \pm 10\%$ greater with 4-amino-pyridine (60 μ M, $n=3$, $P<0.05$ paired *t*-test). These results provide additional support to the view that there are separable populations of synapses with high and low probability of release^{16–19}.

We have found evidence for two classes of central excitatory synapses with a sixfold difference in the probability of release. Because they contribute about equally to transmission, if the quantal size of the two classes is similar then 85% of these synapses have a low p_r . Although it is surprising that most synapses appear to be virtually silent, such synapses may transmit primarily in physiological conditions increasing release such as during bursts of presynaptic activity (as occurs with sensory stimuli) or after local secretion of modulators. Conversion of synapses from low to high p_r (as possibly by induction of long-term potentiation) may render these synapses capable of recreating sensory traces in the absence of externally driven bursts.

Note added in proof: A preliminary report¹⁵ published after this study was initiated and a more complete report²⁰ published since its completion used similar techniques to investigate cultured hippocampal autapses and reached conclusions generally in agreement with those presented here. □

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Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca^{2+} -activated secretion

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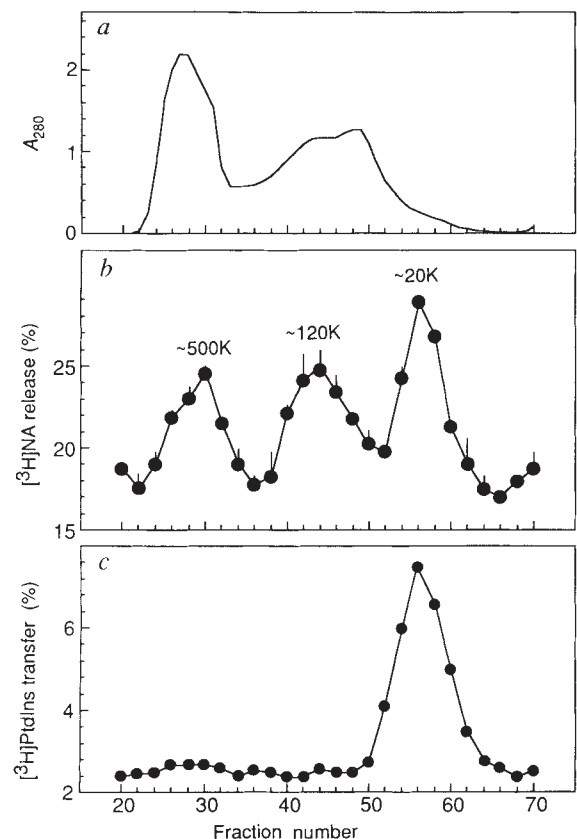
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ELUCIDATION of the reactions responsible for the calcium-regulated fusion of secretory granules with the plasma membrane in secretory cells would be facilitated by the identification of participant proteins having known biochemical activities. The successful characterization of cytosolic^{1–3} and vesicle^{4,5} proteins that may function in calcium-regulated secretion has not yet revealed the molecular events underlying this process. Regulated secretion consists of sequential priming and triggering steps which depend on ATP and Ca^{2+} , respectively, and require distinct cytosolic proteins⁶. Characterization of priming-specific factors (PEP proteins) should enable the ATP-requiring reactions to be identified. Here we show that one of the mammalian priming factors (PEP3) is identical to phosphatidylinositol transfer protein (PITP)⁷. The physiological role of PITP was previously unknown. We also find that SEC14p, the yeast phosphatidylinositol transfer protein which is essential for constitutive secretion^{8–10}, can substitute for PEP3/PITP in priming. Our results indicate that a role for phospholipid transfer proteins is conserved in the constitutive and regulated secretory pathways.

The ATP-dependent priming of Ca^{2+} -activated noradrenaline secretion from permeable PC12 cells was previously found to require proteins present in rat brain cytosol⁶. Gel filtration of rat brain cytosol revealed three size classes of factors with priming activity (ref. 6, and Fig. 1b). These PEP factors (for priming in exocytosis proteins) had apparent molecular masses of ~500,000 (500K) (PEP1), ~120K (PEP2), and ~20K (PEP3). In cytosol from rat liver, only the smallest factor, PEP3, was detectable (not shown); we used this cytosol to purify PEP3 as a 32K protein (Fig. 2b and d). The amino-terminal sequence of the purified PEP3 was VLLKEYRVILPVSVDYQVG-QLYSVA, which is identical to residues 2–27 of the 32K protein predicted from a rat brain complementary DNA encoding PITP¹¹. PITP was originally identified by its ability to transfer

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FIG. 1 Resolution of rat brain cytosolic priming factors by gel-filtration chromatography: *a*, protein; *b*, priming activity; *c*, PtdIns transfer activity. **METHODS.** Rat brain cytosol was prepared and gel-filtered on Superose-12 (Pharmacia) as previously described⁶, except that a modified homogenization buffer was used (20 mM HEPES, pH 7.0, 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, 1.0 mM dithiothreitol (DTT), 1.0 mM PMSF, 2 $\mu\text{g ml}^{-1}$ leupeptin, and 4 $\mu\text{g ml}^{-1}$ aprotinin) and chromatography was run at 1 ml per min with 1-min fractions starting 10 min after injection of sample. Calibration of the Superose-12 column using 10 marker proteins has been described⁶; fraction 1 in ref. 6 corresponds to fraction 21 here. Fractions were assayed for priming activity in cytosol-depleted permeable PC12 cells as described⁶. Briefly, PC12 cells prelabelled with [³H]noradrenaline ([³H]NA) were permeabilized by cell cracking²⁷, preincubated with buffer containing 10 mM EGTA, and washed extensively to remove soluble cellular constituents. Priming activity of column fractions was determined in 30 °C incubations with washed permeable PC12 cells in KGlu buffer (20 mM HEPES, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA, and 0.1% bovine serum albumin) containing 2 mM Mg-ATP. Thirty-minute priming reactions were terminated by chilling, centrifugation at 800g for 1 min and resuspension of permeable cells. Three-minute triggering incubations were conducted in KGlu buffer containing ~0.5 mg ml⁻¹ rat brain cytosol and sufficient CaCl₂ to establish 1 μM free Ca²⁺. Under these conditions, the extent of Ca²⁺-dependent [³H]NA secretion during triggering reflects priming activity⁶. Priming was assayed in duplicate, with the range of values indicated by a bar or by symbol size. PtdIns transfer from [³H]PtdIns-labelled microsomes to phosphatidylinositol/phosphatidylcholine liposomes was assayed according to ref. 7 as modified in ref. 10, except that incubations were for 60 min at 30 °C, and 0.2 ml ice-cold 0.2 M sodium acetate, pH 5.0, in 0.25 M sucrose was used to precipitate microsomes. [³H]PtdIns microsomes were prepared as described⁷.



phosphatidylinositol (PtdIns) from donor to acceptor membranes *in vitro*⁷. The identity between PEP3 and P1TP was confirmed by the co-elution of PtdIns transfer activity with the priming activity and with the 32K protein in the final purification step on Mono-Q (Fig. 2*b-d*). In this step several isoforms of the protein were evident, as previously reported for P1TP¹². Priming activity, PtdIns transfer activity and 32K protein also eluted together in the preceding two purification steps (data not shown). Moreover, P1TP-specific antibody reacted with the purified 32K PEP3 (Fig. 2*e*), as did a P1TP carboxy-terminal peptide-specific antibody (not shown).

Gel-filtered rat brain cytosol showed a single peak of PtdIns transfer activity (Fig. 1*c*) that exactly co-eluted with the ~20K PEP3 priming factor (Fig. 1*b*). Hence the 32K P1TP, which behaves anomalously on gel filtration^{7,13}, accounts for the low-molecular-weight priming factor (PEP3) present in crude brain cytosol. Western blots of gel-filtration fractions revealed that P1TP immunoreactivity was restricted to the 20K peak (not shown), indicating that PEP1 and PEP2 are distinct priming factors and not oligomeric forms of PEP3/P1TP. Each of the PEP factors may promote ATP-dependent priming by acting at a distinct step, but these steps are likely to be in a common overall pathway because of the observed synergism between PEP proteins. Small amounts of PEP3/P1TP, which exerted a minimal effect, were found to potentiate the activity of comparably limiting amounts of the ~500K PEP1 (Fig. 3). One explanation for this super-additivity between PEP proteins is that each factor catalyses an independent reaction in a sequential metabolic pathway.

SEC14p, the P1TP of *S. cerevisiae*, is an essential gene product required for protein secretion from the Golgi to the plasma membrane⁸⁻¹⁰. Mutant *sec14* strains that are temperature-sensitive for secretion show PtdIns *in vitro* transfer activity that is temperature-sensitive supporting a role for phospholipid transfer

in the constitutive secretory pathway *in vivo*⁹. Although mammalian P1TP and SEC14p have no sequence similarity, their biochemical activities are remarkably similar *in vitro*, both catalysing transfer of PtdIns and, to a lesser extent, phosphatidylcholine^{7,14,15}. Purified recombinant SEC14p had substantial ATP-dependent priming activity in permeable PC12 cells (Fig. 4*a*). At submaximal doses, the same number of units of SEC14p or mammalian PEP3/P1TP transfer activity exerted comparable priming activity (Fig. 4*b*). SEC14p promoted priming to a lesser maximal extent than PEP3/P1TP (Fig. 4), but like the mammalian factor, SEC14p activity was markedly synergized by inclusion of a PEP1 fraction (results not shown). The ability of the yeast protein to substitute in mammalian cells suggests that SEC14p and PEP3/P1TP act through a common mechanism in priming, and that this mechanism is used by both the constitutive and regulated secretory pathways. Our observations reinforce the idea that secretory mechanisms are conserved between yeast and mammalian cells⁵, and that universal biochemical mechanisms underlie aspects of constitutive and regulated membrane fusion⁴.

To our knowledge, the identification of PEP3 as P1TP represents the first characterization of an essential factor in the regulated secretory pathway with a known biochemical activity. Recognition of the *in vitro* catalytic activity of PEP3 as phospholipid transfer should facilitate the dissection of the biochemical pathway for ATP-dependent priming. As *in vitro* PtdIns transfer by PEP3/P1TP does not require ATP and its priming activity does, priming may involve the phosphorylation of the recipient membrane of PtdIns to produce polyphosphoinositides. This possibility is consistent with the presence of PtdIns-4-OH kinase as an intrinsic constituent of secretory granules^{16,17}, and with previous work demonstrating a correlation between cellular polyphosphoinositide levels and competence for calcium-regulated exocytosis¹⁸. This mechanism would implicate phosphoino-

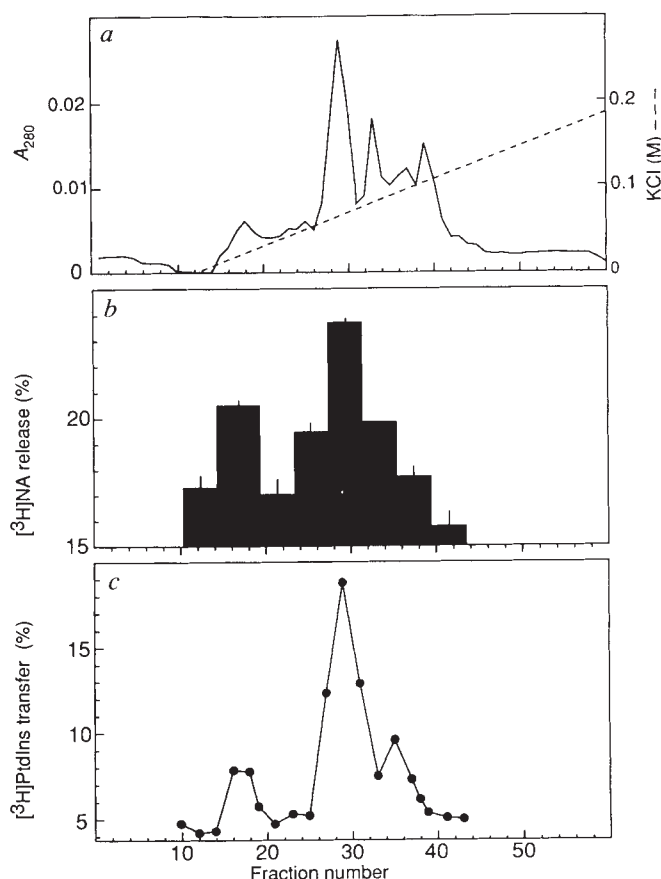


FIG. 2 Purification of PEP3 from rat liver cytosol and its identification as phosphatidylinositol transfer protein. *a*, Elution of protein; *b*, priming activity; *c*, PtdIns transfer activity on Mono-Q; *d*, silver-stained polyacrylamide gel of Mono-Q fractions, numbered as shown (top); migration of M_r markers is shown on the right. *e*, Autoradiogram from immunoblot of Mono-Q fractions with a polyclonal PITP antibody.

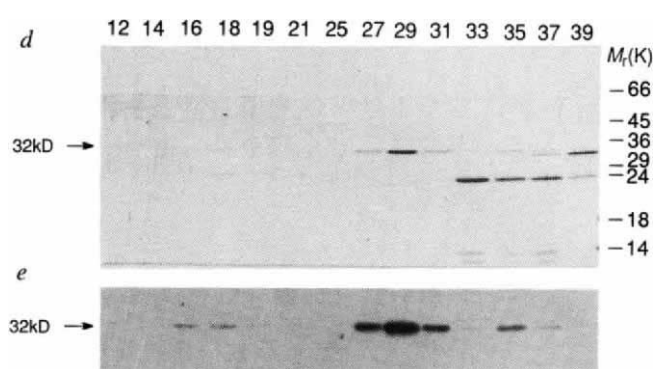


FIG. 3 Synergistic effect of PEP3/PITP on priming by PEP1, the ~500K cytosolic priming factor. Priming activity of PEP1 is shown for the indicated amounts of protein in the presence (circles) or absence (filled circles) of ~13 μ g partially purified PEP3/PITP.

METHODS. Fractions 25–32 from gel filtration of rat brain cytosol shown in Fig. 1, plus those fractions from two identical runs, were pooled and purified on a 2 ml phosphocellulose column (Whatman P11). Phosphocellulose column fractions containing priming activity were strongly potentiated by PEP3/PITP. The priming activity of the partially purified PEP1 is shown in the presence and absence of ~13 μ g gel filtration fraction 56 (PEP3/PITP).

METHODS. Cytosol from five freshly dissected rat livers was prepared as described in Fig. 1 legend for brain. Proteins precipitating at 40–65% saturated ammonium sulphate were collected. The resuspended pellet fraction was adjusted to 1.4 M ammonium sulphate (35% saturation), applied to a 32 cm $\times$ 2.6 cm phenyl-Sepharose column (Pharmacia), and eluted with a reverse ammonium sulphate gradient from 1.4 to 0 M. Priming activity, which eluted at 0 M ammonium sulphate with isocratic washing, was pooled, concentrated, and fractionated by gel filtration on Superose-12 as described for Fig. 1. Priming activity from gel-filtration fractions was pooled and applied to a 100 $\times$ 7.8 mm hydroxylapatite HPLC column (BioRad HPHT), and eluted with a potassium phosphate gradient from 0 to 350 mM. The priming activity eluted at two positions, 40 and 55 mM. Priming activity in the 40 mM peak was pooled, dialysed, applied to a Mono-Q HR 5/5 column (Pharmacia), and eluted with a KCl gradient from 0 to 300 mM. To assay for priming activity as described in Fig. 1 legend, individual column fractions or pools of fractions were concentrated and exchanged into KGLu buffer lacking bovine serum albumin using Centricon-10 devices (Amicon). PtdIns transfer activity of column fractions was assayed as described in Fig. 1 legend. Fractions were resolved by electrophoresis on SDS 12% polyacrylamide gels and analysed by silver staining and western blotting with PITP antibodies as described^{28–30}. For protein sequencing, 19 pmol purified PEP3 was electroblotted onto Problott (Applied Biosystems); the 32K band was excised and sequenced using an Applied Biosystems 470A protein sequencer and 120A online PTH-AA analyser (conducted by W. S. Lane).

side kinases as responsible for the ATP requirement in priming, in a role possibly similar to VPS34 PtdIns kinase in yeast vacuolar protein sorting^{19,20}. Spatially localized synthesis of polyphosphoinositides could serve to regulate interactions between the cytoskeleton and fusing membrane compartments, mediated by phosphoinositide-binding proteins^{21–24}, a suggestion consistent

with the inositol auxotrophy and actin cytoskeletal phenotypes of the *sec14* suppressor *SAC1* in *S. cerevisiae*²⁵.

As other suppressors of *sec14* represent mutations in the phosphatidylcholine biosynthetic pathway involving CDP-choline²⁶, the phosphatidylcholine transfer activity of PEP3/PITP should be considered in elucidating the priming pathway. Alternatively,

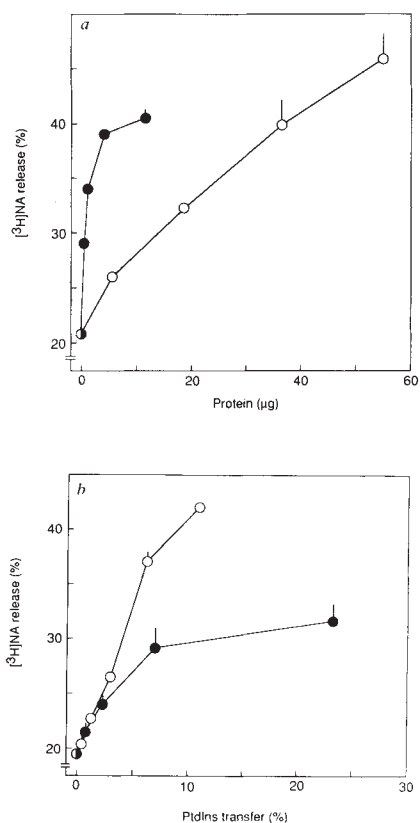


FIG. 4 Priming activity of purified recombinant SEC14p. *a*, Priming activity of SEC14p (filled circles) and partially purified PEP3/PITP (circles) as a function of protein added. *b*, Priming activity of SEC14p (filled circles) and partially purified PEP3/PITP (circles) as a function of PtdIns transfer activity.

METHODS. Purified hexahistidine-tagged SEC14p, expressed in yeast, was provided by V. Bankaitis and H. B. Skinner. Rat brain PEP3/PITP was purified by gel-filtration of cytosol as in Fig. 1. The PtdIns transfer activity of the two fractions (*b*) was measured as described in Fig. 1 legend. Specific PtdIns transfer activities of the fractions were 77% [³H]PtdIns transfer per min per mg for SEC14p, and 2% per min per mg for PEP3/PITP. The maximal extent of priming supported by SEC14p appeared to decrease with storage of the fraction. The experiment shown in *a* was done 9 days before the experiment shown in *b*.

an unknown function for PEP3/PITP in priming, possibly regulated by phospholipid binding⁹, cannot be excluded. Clarification of the identities of PEP1 and PEP2 will provide critical information for establishing the nature of the priming pathway and a molecular basis for the ATP requirement in secretion. □

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Cloning and characterization of an extracellular Ca^{2+} -sensing receptor from bovine parathyroid

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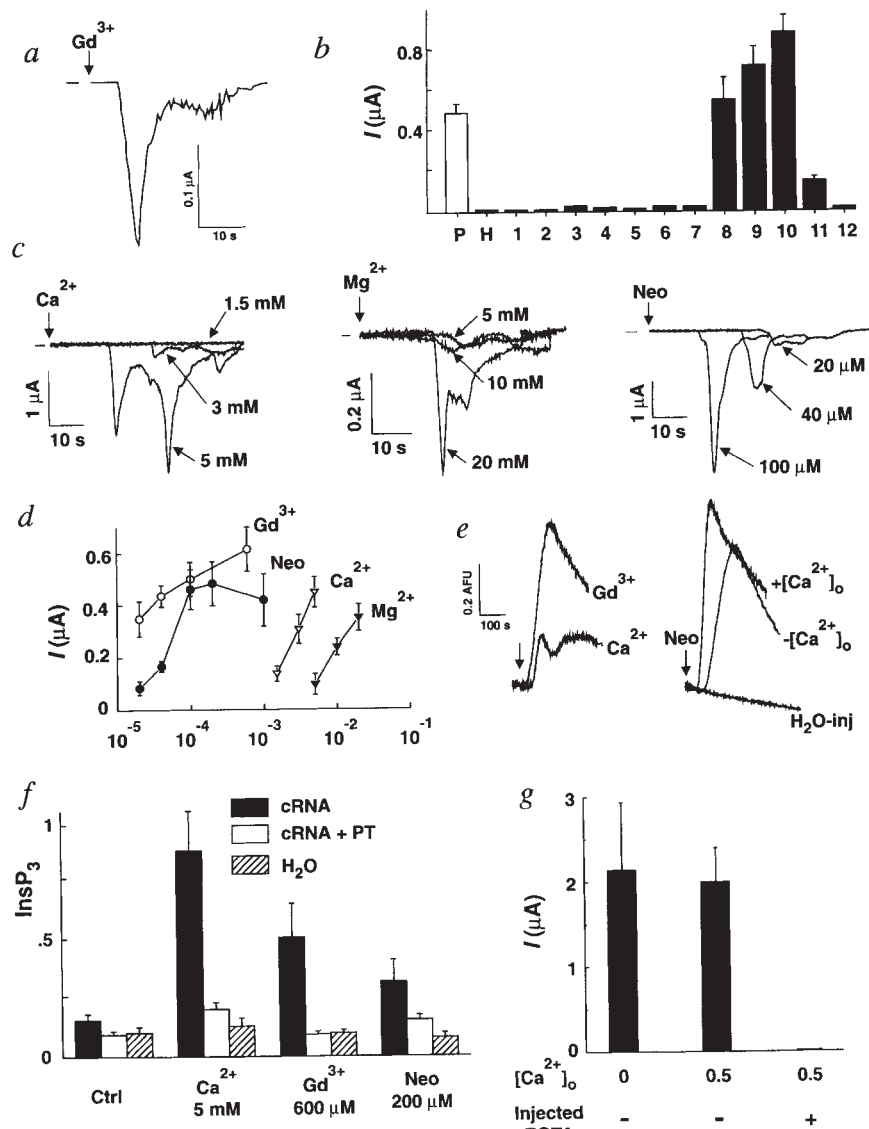
MAINTENANCE of a stable internal environment within complex organisms requires specialized cells that sense changes in the extracellular concentration of specific ions (such as Ca^{2+}). Although the molecular nature of such ion sensors is unknown, parathyroid cells possess a cell surface Ca^{2+} -sensing mechanism that also recognizes trivalent and polyvalent cations (such as neomycin) and couples by changes in phosphoinositide turnover and cytosolic Ca^{2+} to regulation of parathyroid hormone secretion^{1–4}. The latter restores normocalcaemia by acting on kidney and bone². We now report the cloning of complementary DNA encoding an extracellular Ca^{2+} -sensing receptor from bovine parathyroid with pharmacological and functional properties nearly identical to those of the native receptor. The novel ~120K receptor shares limited similarity with the metabotropic glutamate receptors⁵ and features a large extracellular domain, containing clusters of acidic amino-acid residues possibly involved in calcium binding, coupled to a seven-membrane-spanning domain like those in the G-protein-coupled receptor superfamily.

Expression in *Xenopus laevis* oocytes of phosphatidylinositol (PtdIns) coupled receptors produces agonist-dependent inward currents through stimulation of Ca^{2+} -activated Cl^- channels⁶. *X. laevis* oocytes injected with bovine parathyroid poly(A)⁺ RNA respond to extracellular Gd^{3+} (a potent (50% effective concentration, EC_{50} = 35 μM) agonist of the native extracellular Ca^{2+} -sensing receptor^{7,8}) with similar currents (Fig. 1*a*) that are blocked by the Cl^- channel blocker, 9-anthracene carboxylic

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FIG. 1 a, Gd^{3+} -induced (20 μM at arrow) inward current in *X. laevis* oocyte injected with total poly(A)⁺ RNA (line on left represents zero current). b, Gd^{3+} -activated currents in oocytes injected with total mRNA (P), H₂O (H), or mRNA pools (~1.5–7 kb; pools 1–12). c, Current elicited in BoPCaR1 cRNA-injected oocytes by (arrows) Ca^{2+} , Mg^{2+} or neomycin (Neo). Gd^{3+} (20–600 μM) evoked similar responses. d, Dose-responses for Gd^{3+} (○), neomycin (●), Ca^{2+} (▽) or Mg^{2+} (▼) evoked currents in cRNA-injected oocytes (error bars, s.e.m.; $n=9$ –14 oocytes from 3 or more animals). e, Polycations increase [Ca]_i in cRNA-injected oocytes. Left tracings: fluo3 fluorescence (AFU, arbitrary fluorescence units) increased several-fold after exposure (arrow) to Gd^{3+} (300 μM) or Ca^{2+} (5 mM) in medium with 0.5 mM Mg^{2+} and 0.5 mM Ca^{2+} . Right tracings: neomycin (Neo; 1 mM at arrow) increased fluo3 fluorescence similarly with (+[Ca²⁺]_o) or without (–[Ca²⁺]_o); no added Ca^{2+} and 0.1 mM EGTA extracellular Ca^{2+} in cRNA- but not H₂O-injected oocytes (H₂O-inj). f, Polyvalent cations elicit pertussis toxin-sensitive increases in inositol 1,4,5-trisphosphate (pmol per oocyte) in cRNA-injected oocytes ($n=5$ –10). g, Gd^{3+} (500 μM)-evoked inward currents in cRNA-injected oocytes require an increase in [Ca]_i that is not dependent on extracellular Ca^{2+} ; EGTA-injected oocytes were injected with ~100 μM EGTA 15–60 min before study ($n=9$ –16).

METHODS. Isolation of BoPCaR1 cDNA clone: mRNA from calf parathyroid glands was size-fractionated²⁶. A cDNA library was prepared in pSPORT1 (BRL) from 4–6 kb mRNA (b; pools 9 and 10)²⁷. *In vitro* transcribed cRNA from pools of clones was injected into *X. laevis* oocytes and assayed 3–4 days later for currents elicited by Gd^{3+} (20–600 μM)²⁷. Clones from one positive pool were subdivided until the BoPCaR1 clone was isolated. Currents were recorded by two-electrode voltage clamp²⁷ (holding potential, –50 mV) from defolliculated oocytes injected 3–4 days earlier with 50 nl mRNA (0.5 $\mu g \mu l^{-1}$), BoPCaR1 cRNA (0.125 $\mu g \mu l^{-1}$), or H₂O (50 nl). Polycations were added directly to a medium containing (mM): 96 NaCl, 2 KCl, 0.5 CaCl₂, 0.5 MgCl₂, 5 mM HEPES, pH 7.5. [Ca]_i in oocytes was measured using fluo3 (50 pmol per oocyte)²⁸. Inositol



1,4,5-trisphosphate was measured by binding assay (Amersham) in oocytes preincubated with or without 10 $\mu g ml^{-1}$ pertussis toxin for 48 h and then incubated as indicated for 5 min.

acid (9-AC). Functional screening of a directional cDNA library prepared from size-fractionated poly(A)⁺ RNA expressing maximal Gd^{3+} -stimulated Cl^{-} currents (Fig. 1b) led to isolation of a single 5.3-kilobase (kb) clone BoPCaR1 (bovine parathyroid calcium-sensing receptor).

Ca^{2+} , Mg^{2+} , Gd^{3+} and neomycin evoke large inward currents in oocytes injected with complementary RNA prepared from BoPCaR1 (Fig. 1c), with apparent EC_{50} values (about 3 mM, 10 mM, 20 μM and 60 μM , respectively; Fig. 1d) virtually identical to those for the native parathyroid receptor (3 mM⁸, 10–15 mM², 35 μM ^{7,8} and 70 μM ⁹, respectively). Polyarginine, another polycationic agonist in parathyroid cells¹⁰, elicits comparable inward currents in cRNA-injected oocytes ($0.72 \pm 0.02 \mu A$ at 10 $\mu g ml^{-1}$).

Polyvalent cations produce these electrophysiological responses by mobilizing intracellular Ca^{2+} stores through activation of PtdIns-specific phospholipase C (Fig. 1e–g). Ca^{2+} , Gd^{3+} or neomycin evoke increases in the cytosolic Ca^{2+} concentration

FIG. 2 Nucleotide and predicted amino-acid sequences (single-letter code) of BoPCaR1 cDNA. Nucleotides are numbered beginning with the first ATG contained within a strong Kozak consensus sequence²⁹, (AGCAUGG). Stop codons precede this ATG in all frames. Potential membrane-spanning helices (M1–M7) are underlined. The putative signal sequence/peptide (SP) is boxed and the hydrophobic A-segment¹⁵ is underlined (dashed line). Potential phosphorylation sites³⁰ for protein kinase C (◆), N-linked glycosylation sites (▲), and conserved cysteines (*) are labelled. Two of the consensus sites for N-linked glycosylation (Asn 894 and Asn 998) are located in the potential intracellular, C-terminal domain. No consensus sites for protein kinase A phosphorylation are present in the predicted sequence, and cAMP is not known to modulate the function of the extracellular Ca^{2+} -sensing receptor in bovine parathyroid cells². A polyadenylation signal sequence (AATAAA; double underline) precedes the poly(A)⁺ tail. Acidic amino acids (D, E) are circled, and doublets and triplets are underlined with arrows. **METHODS.** Both strands of the BoPCaR1 cDNA were sequenced using Sequenase version 2.0 T7 DNA polymerase (US Biochemical). Geneworks 2.2 (Intelligenetics) was used for sequence analysis and assembly as well as for hydrophathy analysis.

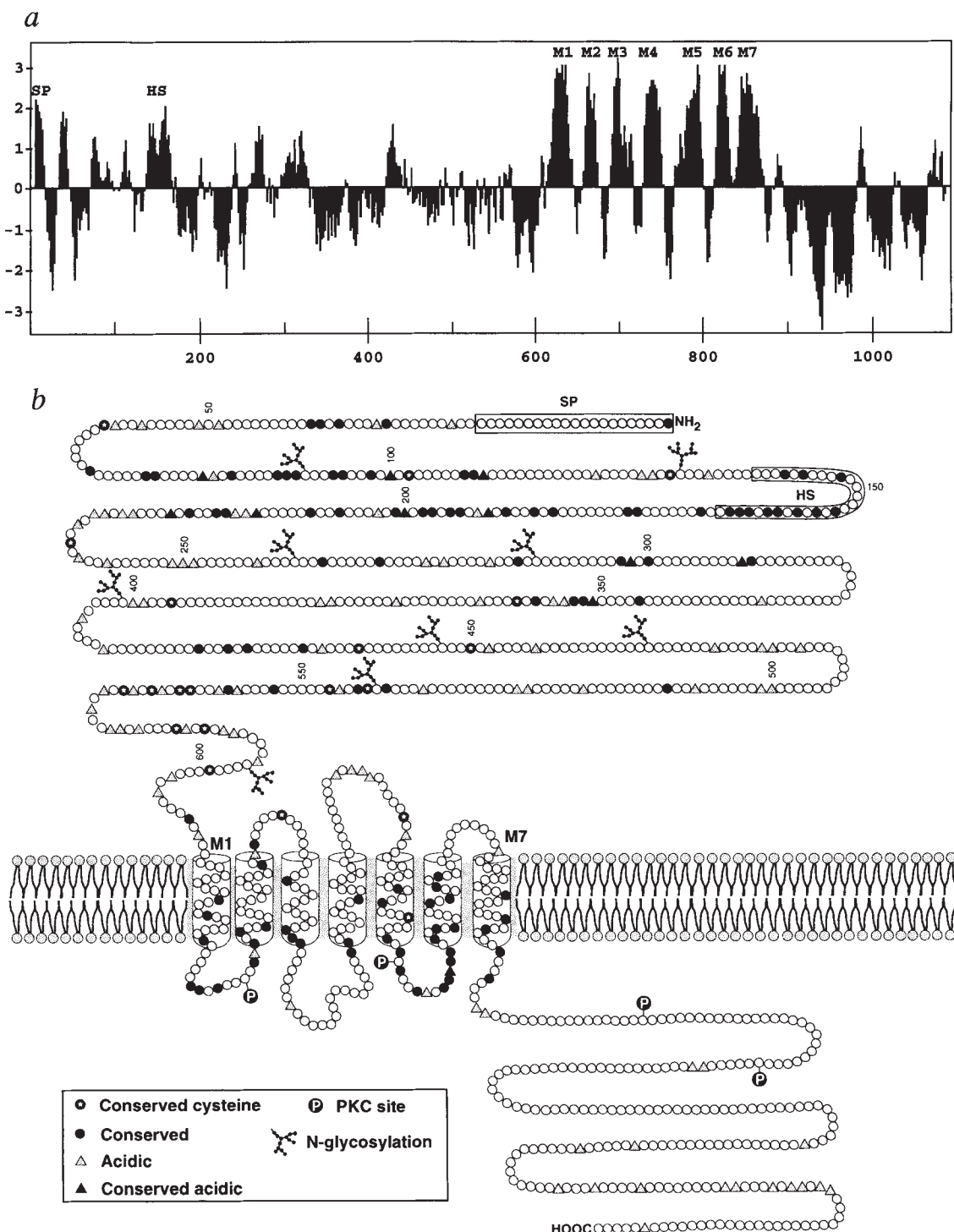
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($[Ca_i]$) that persist in the absence of extracellular Ca^{2+} (Fig. 1e) and result from G-protein-dependent activation of phospholipase C, because Ca^{2+} , Gd^{3+} and neomycin produce pertussis toxin(PT)-sensitive, 4–7-fold increases in inositol 1,4,5-trisphosphate levels (Fig. 1f). Like other receptors, therefore, BoPCaR1 apparently couples to PLC in *X. laevis* oocytes through G proteins differing in PT-sensitivity from those used by the receptor in native cells^{11,12}. The inhibition of Gd^{3+} -activated inward currents by 9-AC and by intracellularly injected EGTA, but not by removal of extracellular Ca^{2+} (Fig. 1g), is consistent with currents arising from activation of Cl^- channels by mobiliz-

ation of internal Ca^{2+} stores. These results also suggest that the BoPCaR1 protein is not a Ca^{2+} channel or transporter.

The 5,275-base-pair (bp) BoPCaR1 cDNA has a 3,255-bp open reading frame encoding a protein of 1,085 amino acids (M_r 121,033) with three structural domains (Figs 2 and 3): a large, predominantly hydrophilic amino-terminal domain of 613 amino acids containing an initial hydrophobic, 21-amino-acid segment characteristic of eukaryotic signal sequences¹³ and 9 potential N-linked glycosylation sites, suggesting an extracellular location; a central core of 250 amino acids containing seven potential membrane-spanning helices (M1–M7) characteristic of

FIG. 3 a, Hydropathy plot for the deduced BoPCaR1 protein sequence. Hydropathy values²⁷ are plotted against amino-acid position using a window of 10. Positive values indicate hydrophobic regions and negative values correspond to hydrophilic regions. Regions corresponding to predicted transmembrane segments (M1–M7) are shown, as are the hydrophobic signal peptide (SP) and hydrophobic A-segment (HS). b, Proposed structural model for the predicted BoPCaR1 Ca^{2+} -sensing receptor protein. The large N-terminal domain is located extracellularly and contains 9 potential N-linked glycosylation sites (shown as branched chains). Amino acids are indicated at intervals of 50 in the N-terminal domain. Amino-acid segments forming each of the seven putative membrane spanning helices are numbered. Potential phosphorylation sites for PKC are shown. Symbols represent individual amino acids. Amino acids that were identical or in the following groups in all mGluRs and BoPCaR1 are shown: acidic, (▲) D, E; non-acidic (●) {M, I, L, V}, {F, Y, W}, {A, G}, {S, T}, {Q, N} and {K, R, H}.



the G-protein-coupled receptor (GPR) superfamily¹⁴; and a hydrophilic, 222-amino-acid-containing carboxy terminus predicted to be cytoplasmic. Glycosylation of the BoPCaR1 protein was confirmed on *in vitro* translation (Fig. 4a). The native extracellular Ca^{2+} -sensing receptor on bovine parathyroid cells also appears to be glycosylated².

The proposed topology of the BoPCaR1 Ca^{2+} -sensing receptor, with its large putative extracellular domain (Fig. 3b), is similar to that predicted for the metabotropic glutamate receptors (mGluRs)^{5,15-18} and the glycoprotein hormone receptors¹⁹. The BoPCaR1 receptor shares limited but significant overall homology with the mGluRs (19–25% identity, 25–30% similarity for mGluR1–6), but not with the glycoprotein hormone receptors or other GPRs. The highest degree of similarity is to mGluR1 (refs 15, 18) and mGluR5 (ref. 17) which also have long C-terminal domains and couple to PtdIns hydrolysis in *Xenopus* oocytes. A common ancestry for BoPCaR1 and the mGluRs is also suggested by the striking conservation of several localized regions. BoPCaR1 shares with the mGluRs the relative positions of 20 cysteines (seventeen in the N-terminal domain, one each in the first and second predicted extracellular loops, and one in M5; Figs 2 and 3b). In addition, a hydrophobic segment (amino acids 138–161; Fig. 2) in the N terminus of BoPCaR1 is clearly similar (50% similarity, 38% identity) to the hydrophobic, N-terminal A segment in mGluRs¹⁵. The conserved cysteines and the A segment may provide a structural framework for ligand interaction in the mGluRs¹⁶ and could serve a similar function in BoPCaR1. Moreover, segments comprising the putative first and third intracellular loops (Figs 2 and 3b; amino acids 637–650 and 794–806) are significantly conserved in BoPCaR1 and the mGluRs (43% and 62% identities, respectively) but exhibit no significant homology to other GPRs. Although these loop segments in the GPR superfamily are thought to be involved in G-protein interaction¹⁴, the role of these highly conserved¹⁶ loops in G-protein interactions in the mGluRs has been questioned because of the variety of signal transduction pathways linked to these receptors²⁰. Of potential importance, however, the first and third cytoplasmic loops and the cytoplasmic C terminus contain predicted phosphorylation sites for protein kinase C (PKC) (Figs 2 and 3). Because activators of PKC attenuate the high Ca^{2+} -induced increases in $[\text{Ca}]$ and inositol phosphates in parathyroid cells², the predicted PKC phosphorylation sites on the BoPCaR1 protein could play a regulatory role.

Despite these conserved structural features, the overall similarity between BoPCaR1 and the mGluRs is less than the ~43–70% homology between individual mGluRs¹⁶. This divergence is also supported by the distinct pharmacology of BoPCaR1, namely the sensitivity to extracellular Ca^{2+} and other polyvalent cations (Fig. 1c–g). Moreover, L-glutamate and the mGluR-specific agonist, 1S, 3R-ACPD²⁰, fail to activate inward currents in BoPCaR1-injected oocytes. This novel pharmacology prompted a search for consensus Ca^{2+} -binding sites. The low apparent affinity of BoPCaR1 for extracellular Ca^{2+} (~3 mM; Fig. 1c) is consistent with the lack of consensus sequences for higher-affinity Ca^{2+} -binding sites, such as EF hands^{21,22}, epidermal growth factor-like repeats²², or Ca^{2+} -dependent carbohydrate binding sites²³. Highly acidic regions, however, have been implicated in the binding of Ca^{2+} to low-affinity (dissociation constant, K_d , of the order of millimolar Ca^{2+} -binding proteins, such as calsequestrin²⁴. Two regions (amino acids 216–251 and 557–611; Figs 2 and 3a) in the putative extracellular N-terminal domain and a short acidic segment in the second extracellular loop (ELEDE; Fig. 2) of BoPCaR1, but not mGluRs, contain a high density of acidic residues (29–38%), some present as doublets and triplets. These acidic regions could potentially be involved in the binding of Ca^{2+} and other polyvalent cations to BoPCaR1.

On northern analysis (Fig. 4), bovine parathyroid, kidney cortex and outer medulla, thyroid, and some regions of brain

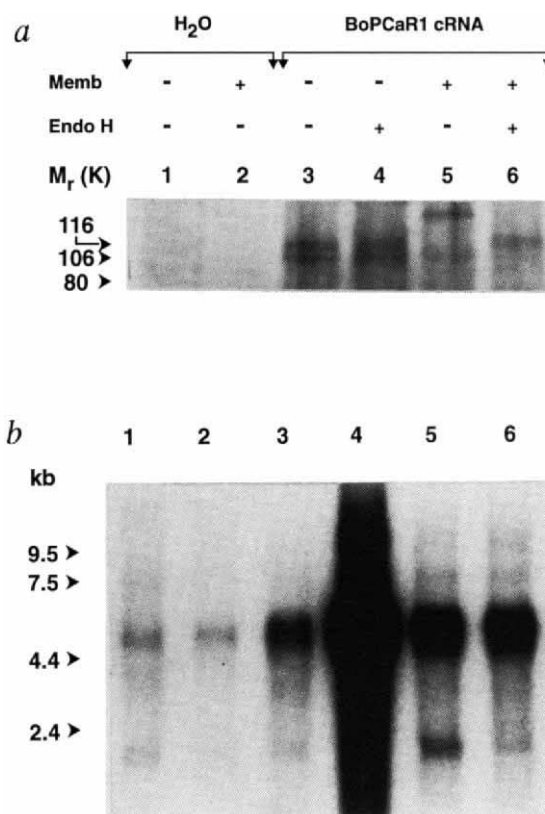


FIG. 4 a, The *in vitro* translated BoPCaR1 protein can be glycosylated (lanes 1 and 2, no cRNA, H_2O control; lanes 3–6, BoPCaR1 cRNA with and without membranes (M) and/or endoglycosidase H (Endo H)). Note the increase in apparent M_r , on translation in the presence of microsomes (~115 to ~135K), which is reversed by enzymatic deglycosylation. These BoPCaR1 protein products are of considerably lower M_r than the 500K protein recognized by a monoclonal antibody that has been postulated to interact with a Ca^{2+} sensor in placenta⁴. b, Tissue distribution of the BoPCaR1 Ca^{2+} -sensing receptor. Northern hybridization of poly(A)⁺ RNA (5 μg) from bovine cerebral cortex (lane 1), cerebellum (lane 2), thyroid (lane 3), parathyroid (lane 4), kidney cortex (lane 5), and kidney outer medulla (lane 6). No signal was detected in cardiac atrium and ventricle, lymph node, thymus, adrenal gland, ureter, urinary bladder, aorta, inferior vena cava, lung, skeletal muscle, stomach, oesophagus, liver, gallbladder and spleen.

METHODS. BoPCaR1 cRNA was translated *in vitro* with or without canine pancreatic microsomes, and translation products were treated with endoglycosidase H as described²⁷. Protein products were resolved by Laemmli SDS–polyacrylamide gel electrophoresis. Northern blots were done at moderate stringency (wash; 50 °C, 0.5 × SSCP, 0.1% SDS) using an antisense ³²P-labelled randomly primed probe with an exposure time of 1 week²⁷. Fainter bands were seen in these tissues at high stringency (65 °C; 0.1 × SSCP, 0.1% SDS).

contain transcripts similar in size (~5.3 kb) to the BoPCaR1 cDNA. Thus the direct effects of extracellular Ca^{2+} (and Mg^{2+}) on renal proximal tubule, medullary thick ascending limb of Henle's loop and thyroidal C cells^{2,7} might be mediated by extracellular Ca^{2+} -sensing receptors similar to BoPCaR1. In other tissues expressing transcripts for BoPCaR1, such as brain, extracellular Ca^{2+} -sensing may play previously unrecognized roles in regulating neuronal function or in modulating hormones involved in calcium homeostasis.

Thus, we propose that BoPCaR1 in parathyroid cells (and BoPCaR1-like receptors in other tissues) provides the essential extracellular Ca^{2+} -sensing mechanism used in regulating calcium metabolism². Moreover, the capacity of the extracellular Ca^{2+} -sensing receptor to recognize neomycin could potentially

underlie some of the toxic effects of aminoglycoside antibiotics²⁵ on cortical and outer medullary regions of the kidney. Finally, analogous extracellular ion-sensing receptors could exist (for example, for K^+ and PO_4^{3-}), which might provide not only a mechanism for inorganic ions directly affecting cellular function², but also, the extracellular ion-sensors involved in maintaining their respective extracellular ion concentrations. □

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Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins

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BINDING of interferons IFN- α and IFN- γ to their cell surface receptors promptly induces tyrosine phosphorylation of latent cytoplasmic transcriptional activators^{1–4} (or Stat proteins, for signal transducers and activators of transcription). Interferon- α activates both Stat91 (*M*, 91,000; ref. 1) and Stat113 (*M*, 113,000; ref. 2) whereas IFN- γ activates only Stat91 (refs 3, 4). The activated proteins then move into the nucleus and directly activate genes induced by IFN- α and IFN- γ . Somatic cell genetics experiments have demonstrated a requirement for tyrosine kinase-2 (Tyk2) in the IFN- α response pathway⁵ and for Jak2 (ref. 6), a kinase with similar sequence⁷, in the IFN- γ response pathway. Here we investigate the tyrosine phosphorylation events on Stat and Jak proteins after treatment of cells with IFNs α and γ and with epidermal growth factor (EGF). Stat91 is phosphorylated on Tyr 701 after cells are treated with IFN- α and EGF, as it was after treatment with IFN- γ (ref. 8). We find that Jak1 also becomes phosphorylated on tyrosine after cells are treated with these same three ligands, although each ligand is shown to activate at least one other different kinase. Jak1 may therefore be the enzyme that phosphorylates Tyr 701 in Stat91.

Although IFN- α and IFN- γ bind to structurally different receptors, Stat91 is activated by both ligands^{3,4}, suggesting that Stat91 might also be activated by other ligand-receptor interactions. EGF activates a transcriptional factor SIF (*c-sis*-inducible factor), which binds the DNA element SIE (*c-sis*-inducible element) in the *c-fos* promoter. An artificial oligonucleotide sequence that has a higher affinity than the natural site for SIF binding was used in most of these studies^{9–11}. This sequence,

CATTCCCGTAAATC, is very similar to the GAS site (IFN- γ -activation site, consensus TTNCNNNA) to which Stat91 binds^{12–15}, suggesting that SIF and Stat91 may be related. To test whether Stat91 is activated through the EGF receptor, anti-91T was used to immunoprecipitate extracts of A431 cells (human cells carrying many EGF receptors) before and after EGF treatment. Immunoprecipitated protein was resolved by SDS-PAGE and tested by western blotting for tyrosine phosphorylation with a monoclonal anti-phosphotyrosine antibody. Stat91 tyrosine phosphorylation was detected within 1 min of EGF stimulation of the cells. In contrast, Stat113 was not phosphorylated on tyrosine in response to EGF (Fig. 1a). Binding of EGF as well as IFN- α and - γ to their respective receptors therefore leads to phosphorylation of Stat91 on tyrosine.

After IFN- γ activation, a single residue, namely Tyr 701, is phosphorylated in Stat91 (ref. 8). To determine whether Stat91 is phosphorylated on the same residue in response to EGF and IFN- α , we labelled A431 cells and FS2 (fibroblast) cells with ³²P-orthophosphate and treated them with IFN- α , - γ or EGF. Stat91 was collected by immunoprecipitation (Fig. 1b) and analysed by two-dimensional tryptic peptide mapping (Fig. 1c; refs 3, 4, 8). A single phosphotyrosine-containing peptide, labelled X, was detected after IFN- γ treatment from either A431 (Fig. 1c, C) or FS2 cells (Fig. 1c, E). This corresponds to the Tyr 701 peptide⁸. This same phosphopeptide was also present in cells treated with EGF and IFN- α (Fig. 1c, B and F, respectively). Mixing of ³²P-labelled peptides from IFN- γ -treated cells and that from either IFN- α -treated cells or from EGF-treated cells followed by reanalysis (Fig. 1c, D and G) yielded a similar pattern of tryptic maps. Thus IFN- α , IFN- γ and EGF all lead to phosphorylation of Stat91 on Tyr 701, so Stat91 may be the substrate for the same or a similar kinase triggered by each of the three ligands.

The Stat91 protein has a sequence from Tyr 573 to Asp 674 that resembles SH2 domains¹⁶, amino-acid regions known to bind tightly to tyrosine phosphates¹⁷. As ligand-activated kinases often present a phosphotyrosine to a substrate, we tested the requirement for the SH2 domain in Stat91 in ligand-mediated phosphorylation of Stat91. The Arg 155 residue in the *v-src* SH2 domain is crucial for direct interaction between a phosphotyrosine residue in the *v-src* SH2 domain^{18,19} and Arg 602 of Stat91 is in a comparable position within the SH2 homology domain¹⁶. We therefore changed the Stat91 complementary DNA to encode Leu 602 instead of Arg 602 and inserted the new sequence into an expression vector. U3A cells, a cell line which is unresponsive to IFN- α and IFN- γ ²⁰ and lacks the mRNA for Stat91/84 proteins²¹, were transfected with expression vectors. Two stable cell lines were selected that expressed the

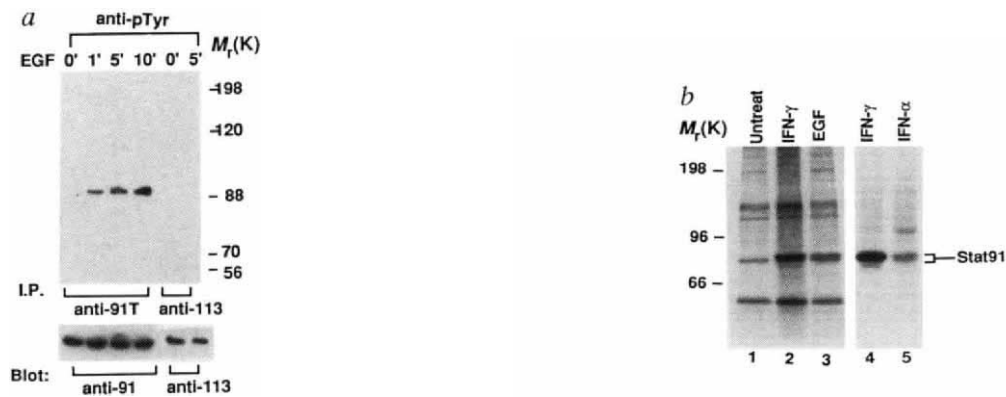
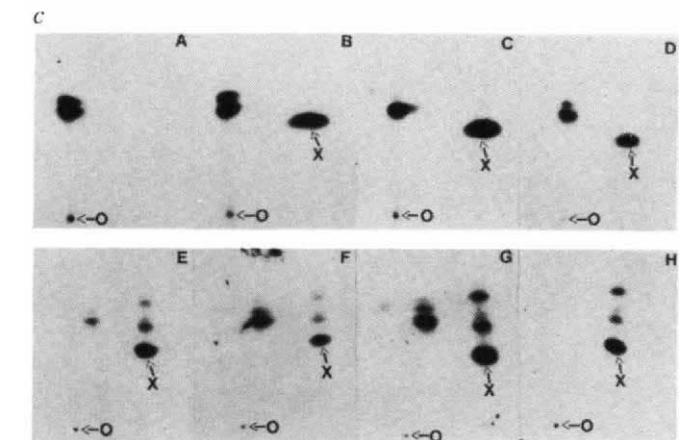


FIG. 1 Phosphorylation of Stat91 on Tyr 701 after IFN- α and EGF. **a**, Whole-cell lysates⁴ from A431 cells treated with EGF (100 ng ml⁻¹) for the indicated times were immunoprecipitated with anti-91T (ref. 4) (lanes 1–4) or anti-113 (lanes 5, 6), separated on 7.5% SDS-PAGE and adsorbed to nitrocellulose. The blot was then probed with a monoclonal antiphosphotyrosine antibody 4G10 (UBI). The blot was stripped and reprobed with anti-91 or anti-113 as indicated (lower panel). **b**, ³²P-labelled A431 (lanes 1–3) or FS2 (fibroblasts) cells⁴ (lanes 4, 5) were treated with IFN- α , IFN- γ or EGF for 10 min. Extracts were precipitated with anti-91T antibody and subjected to 7.5% SDS-PAGE and autoradiography. The position of Stat91 is shown. **c**, Tryptic peptide mapping⁸ of Stat91 from **b**. A, untreated A431; B, EGF-treated A431; C, IFN- γ -treated A431; D, mixture of B and C; E, IFN- γ -treated FS2 cells; F, IFN- α -treated FS2 cells; G, mixture of E and F; H, peptide X from E rerun. Peptide X has the sequence GTGYIK (ref. 8). In analyses E–H, the appearance of minor spots of variable intensity above peptide X in the autoradiograms of the Stat91 tryptic maps was due to experimental artefacts, because authentic peptide X (in E) gave the same spots when rerun by itself (H). Such spots are not reproducible and varied between experiments (see panel C, for example, and refs 3, 8 and 28).

Arg 602→Leu 602 mutant protein, clones cr1 and cr2 (Fig. 2), as were cells expressing wild-type protein. The mutant protein immunoprecipitated from these cell lines was not phosphorylated on tyrosine in response to IFN- γ (Fig. 2b); therefore a functional SH2 domain is required for tyrosine phosphorylation of Stat91 protein. This suggests that the kinase to which the substrate binds might in its active state have a tyrosine phosphate, so we searched for kinases showing ligand-dependent tyrosine phosphorylation.

Tyk2 is required for the IFN- α response and is phosphorylated on tyrosine in response to IFN- α treatment^{5,22}. But cells that lack Tyk2 respond normally to IFN- γ (ref. 23), suggesting a different kinase function in the IFN- γ pathway. Two kinases with sequence similarity to Tyk2 are known, namely Jak1 and Jak2 (ref. 7). It is interesting that Jak1 messenger RNA levels are increased in IFN- γ -treated cells²⁴, hence we determined whether



Jak1 was phosphorylated on tyrosine in response to IFN- γ . Jak1 was immunoprecipitated from extracts of untreated and IFN- γ -treated FS2 cells with an antiserum prepared against a fusion protein containing the first kinase-like domain (amino acids 566–865) of Jak1 (ref. 7). The immunoprecipitates were subjected to SDS-PAGE and western blot analysis using a phosphotyrosine antibody. A protein of M_r ~130K, similar to the reported size of Jak1 (ref. 7), was detected. Jak1 was clearly phosphorylated on tyrosine in response to IFN- γ (Fig. 3a). Reprobing of the same blot with an antiserum directed against a C-terminal Jak1 peptide showed that total Jak1 levels were comparable in treated and untreated cells (Fig. 3a, lanes 3 and 4). As Stat91 was phosphorylated on Tyr 701 after treatment with IFN- α and EGF as well as with IFN- γ , we examined Jak1 tyrosine phosphorylation in response to these other ligands. Figure 3b and c shows that Jak1 was phosphorylated on tyrosine after treatment with each

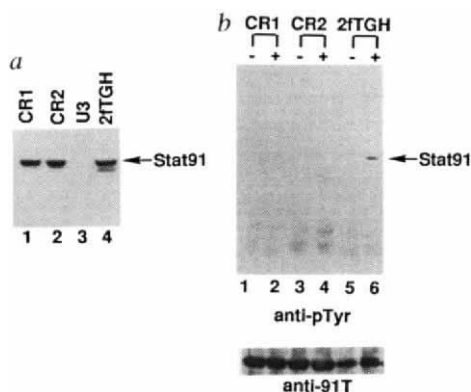
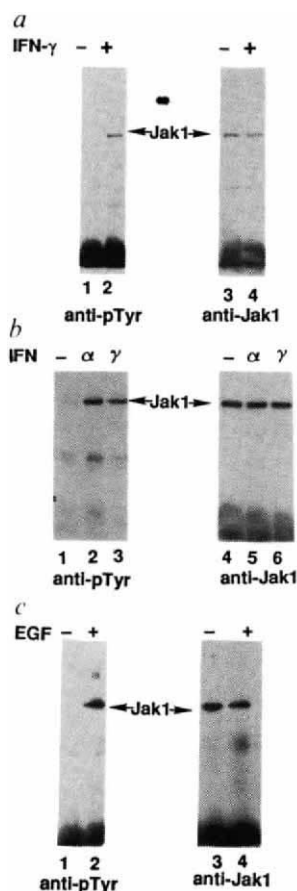


FIG. 2 Residue R 602 in Stat91 SH2 domain is required for tyrosine phosphorylation. **a**, Western blot analysis of whole-cell extracts from mutant U3A cell line (lane 3); parental 2fTGH cell line (lane 4); or U3A-derived cell lines transfected with an expression vector⁸ containing an R 602→L 602 mutation (lanes 1 and 2). Antibody used was anti-91, which recognizes both Stat91 and Stat84 proteins^{1,3}. **b**, Immunoprecipitates with anti-91T antibody were subjected to 7.5% SDS-PAGE and probed with an anti-phosphotyrosine antibody as described for Fig. 1. Mutagenesis was carried out by standard polymerase chain reaction procedure²⁹. The CGG codon for Arg 602 was mutated to CTG, which encodes Leu. Transfection and selection of stable cell lines was described⁸.

FIG. 3 IFN- α , - γ and EGF trigger phosphorylation on tyrosine of Jak1. *a*, FS2 cells, *b*, B lymphocytes (Namalva cells); or *c*, A431 cells were treated with ligands for 10 min as indicated, extracts prepared and precipitated with anti-Jak1, which was raised against amino acids 526–850 of the human Jak1 protein fused to glutathione-S-transferase (GST) (ref. 7). Precipitates were separated on 7.5% SDS-PAGE and probed with an anti-phosphotyrosine antibody as described in Fig. 1 legend. The blots were stripped and blotted with an antiserum raised against a C-terminal peptide of human Jak1 protein⁷.



of the three ligands. Thus each ligand that caused phosphorylation on Tyr 701 of Stat91 also caused tyrosine phosphorylation of Jak1.

Another member of this kinase family, Jak2, was recently shown to associate with the receptor for erythropoietin (Epo) and to become phosphorylated on tyrosine following Epo treatment²⁵. Jak2 phosphorylation was also observed following treatment with interleukin-3 (IL-3) (ref. 26) and IFN- γ (ref. 6). Furthermore, a mutant cell line deficient in response to IFN- γ but not IFN- α could be complemented by a Jak2 cDNA⁶. For these reasons, we compared phosphorylation of Jak1 and Jak2 in cells treated with IFN- α , IFN- γ , EGF and Epo.

Extracts of human 2fTGH cells treated or untreated with IFN- γ were precipitated with antiserum against Jak2 or Jak1 and analysed for phosphorylation. Both Jak1 and Jak2 were phosphorylated on tyrosine in response to IFN- γ treatment (Fig. 4a, top). When the immunoblot was stripped and reprobed with either anti-Jak2 antiserum or the Jak1 antipeptide antiserum (Fig. 4a, bottom left), or with a mixture of the two antisera (Fig. 4a, bottom right), it was clear that each antiserum recognized a protein with distinct electrophoretic mobility. This observation, together with evidence for differential phosphorylation discussed later, suggests strongly that the antisera recognize two different proteins, presumably Jak1 and Jak2.

Although both Jak1 and Jak2 are phosphorylated following treatment with IFN- γ , this is not the case after treatment with Epo²⁵ and IL-3 (ref. 26; J. Ihle and I. Kerr, personal communication), which results in phosphorylation of Jak2 but not Jak1. Furthermore, Epo and IL-3 treatments did not result in Stat91 phosphorylation either, suggesting that Stat91 phosphorylation correlates with Jak1 and not Jak2 phosphorylation. We therefore examined Jak2 phosphorylation in response to IFN- α and EGF, which both trigger phosphorylation of Jak1 and Stat91. Figure 4b shows that, unlike Jak1 and Stat91, Jak2 is not phosphorylated under these conditions.

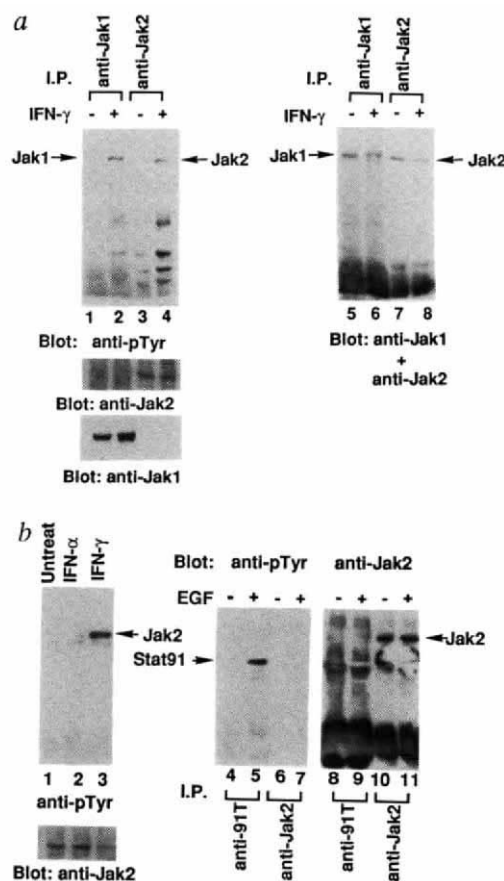


FIG. 4 Jak2 is tyrosine-phosphorylated after IFN- γ but not after IFN- α or EGF. *a*, Extracts of 2fTGH cells, treated as indicated with IFN- γ , were precipitated either with anti-Jak1 (as for Fig. 3) or anti-Jak2 (refs 25, 26; antiserum was a gift of J. Ihle) antisera; precipitates were subjected to 7.5% SDS-PAGE and probed with an anti-phosphotyrosine antibody (top left). The filter was stripped and reblotted either with anti-Jak1 or Jak2 antibodies (insets under upper left) and stripped and reprobed a third time with a mixture of Jak1 and Jak2 antisera (top right). *b*, Human B lymphocytes (NAM) (lanes 1–3) and A431 cells (lanes 4–11) were treated with IFN- α , - γ or EGF as indicated. Extracts were immunoprecipitated with anti-Jak2 (lanes 1–3, 6, 7, 10, 11) or with anti-91T (lanes 4, 5, 8, 9) and analysed on 7.5% SDS-PAGE. The blot was probed with an anti-phosphotyrosine antibody (lanes 1–7) and stripped and reprobed with anti-Jak2 antiserum (lanes 8–11) as indicated.

Thus there is a correlation in all experiments so far: ligands that activate Jak1 lead to phosphorylation of Stat91. After these experiments, mutant cells (U4 cells) that are unresponsive to both IFN- α and IFN- γ were found to lack Jak1 (ref. 22). A cDNA encoding Jak1 restores responsiveness to both IFN- α and IFN- γ . Thus both the genetic and immunochemical data reveal a role for Jak1 in interferon signalling. But it appears that two kinases function in each pathway leading to Stat91 phosphorylation: IFN- γ activates both Jak1 and Jak2, IFN- α activates Tyk2 as well as Jak1, and EGF also brings about the phosphorylation of its own receptor plus Jak1.

How could multiple proteins participate in the ligand-specific activation of latent cytoplasmic transcription factors? As the Jak2 receptor is physically associated with the Epo and growth hormone receptors^{25,27}, it is likely that each specific receptor and its associated kinase form a specific multiprotein complex; this complex could then embody the specificity inherent in selecting and phosphorylating a particular set of Stat proteins. □

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Interferon-induced nuclear signalling by Jak protein tyrosine kinases

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INTERFERONS IFN- α/β and IFN- γ act through independent cell-surface receptors, inducing gene expression through tyrosine phosphorylation of cytoplasmic transcription factors^{1–5}. IFN- α stimulates phosphorylation and nuclear localization of the 84/91K and 113K subunits of latent ISGF3 (interferon-stimulated gene factor 3), which combine with the 48K DNA-binding subunit^{6,7} to bind regulatory elements of IFN- α -responsive genes^{8–10}. IFN- γ activates p91 alone², inducing IFN- γ -responsive genes through a distinct DNA element^{11,12}. Genetic complementation studies implicated the tyrosine kinase Tyk2 in IFN- α signalling¹³ and, more recently, the related Jak2 kinase in IFN- γ signalling¹⁴. We now present biochemical evidence for Jak-family kinase involvement in IFN signal transduction. Jak1 was activated in response to IFN- α and IFN- γ ; Jak2 responded exclusively to IFN- γ . Overexpression of either Jak1 or Jak2 stimulated p91 DNA-binding activity and p91-dependent transcription. Overexpression also activated endogenous Jak kinases, suggesting that interactions between Jak kinases are required during interferon signalling.

Interferon-dependent tyrosine phosphorylation of Jak1 and Jak2 was examined in mouse 3T3 cells treated for various times with IFN- α or IFN- γ (Fig. 1a). IFN- γ stimulation resulted in time-dependent tyrosine phosphorylation of Jak2, with peak

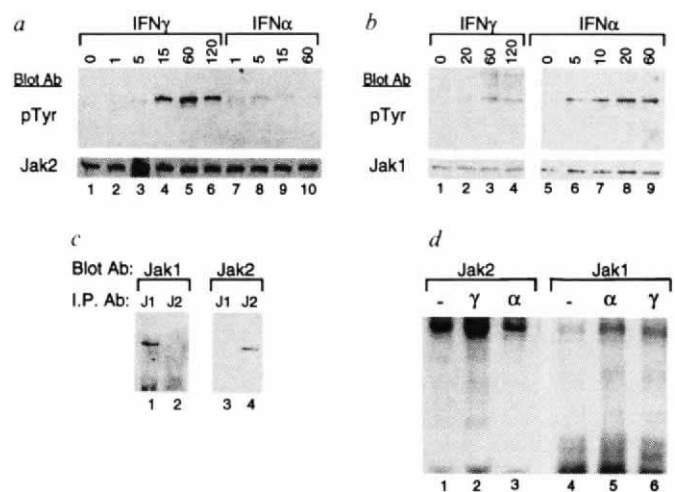


FIG. 1 Tyrosine phosphorylation of Jak1 and Jak2 in response to IFNs. **a**, IFN- γ induces tyrosine phosphorylation of Jak2. Cells were unstimulated or stimulated for the indicated times (min) with either IFN- γ (lanes 2–6) or IFN- α (lanes 7–10). Cell lysates were immunoprecipitated with anti-Jak2 and immunoblotted with anti-phosphotyrosine antibodies (upper panel). The filter was stripped and reblotted with anti-Jak2 (lower panel). **b**, Both IFN- γ and IFN- α stimulate tyrosine phosphorylation of Jak1. Cells were either untreated or treated with IFN- γ or IFN- α for the indicated times, and the cell lysates were immunoprecipitated with anti-Jak1 antibodies for anti-phosphotyrosine blotting (upper panel) and anti-Jak1 reblotting (lower panel). **c**, Specificity of anti-Jak1 and anti-Jak2 antisera. Anti-Jak1 (lanes 1, 3) or anti-Jak2 (lanes 2, 4) immunoprecipitates immunoblotted with anti-Jak2 sera (lanes 1, 2). After stripping, the filter was probed with anti-Jak1 sera (lanes 3, 4). **d**, The effect of IFNs on the *in vitro* kinase activity of Jak1 and Jak2. Cells were either untreated or treated with IFN- α or IFN- γ for 5 min. The cell lysates were immunoprecipitated with anti-Jak2 (lanes 1–3) or anti-Jak1 (lanes 4–6) and analysed for kinase activity as described¹⁸.

METHODS. Serum-starved Swiss 3T3 cells were treated for the indicated times with murine IFN- γ (5 ng ml⁻¹) (**a**, **b**) or (1 ng ml⁻¹) (**d**) or with murine IFN- α/β (1,000 U ml⁻¹) (**a**, **b**); or (500 U ml⁻¹) (**d**). Cells were lysed with Triton X-100, run on SDS-PAGE and transferred to nitrocellulose. Immunoblots were developed with enhanced chemiluminescence (Amersham) according to standard procedures. Anti-Jak1 antisera were raised against a synthetic peptide corresponding to amino acids 118–132 of murine Jak1 (O.S., B. Witthuhn, T. Yi and J. Ihle, manuscript in preparation); anti-Jak2 antisera were raised against amino acids 19–33 of murine Jak2 (ref. 17). Similar results were obtained with anti-Jak2 antisera specific for amino acids 805–819. Commercial monoclonal anti-phosphotyrosine antibodies were obtained from Upstate Biotechnology, ICN and Oncogene Science.

phosphorylation between 20 min and 1 h (Fig. 1a, lanes 1–6 and data not shown). No significant change in Jak2 abundance was observed (Fig. 1a, lower panel). IFN- α , in contrast, did not induce significant tyrosine phosphorylation of Jak2 (Fig. 1a, lanes 7–10).

In contrast to Jak2 activation, both IFN- α and IFN- γ treatment induced tyrosine phosphorylation of Jak1, with differing kinetics in response to each ligand (Fig. 1b). Tyrosine phosphorylation of Jak1 was detected within 5 min of both IFN treatments, but IFN- α -induced phosphorylation was maximal within 20 min whereas the response to IFN- γ increased for 1 h, with no change in protein abundance (Fig. 1b, lower panel). As shown in Fig. 1c, Jak1 and Jak2 antisera did not crossreact with Jak2 or Jak1 protein, respectively, or with the Tyk2 protein (data not shown). Further support for the involvement of Jak kinases in IFN signalling was obtained from *in vitro* kinase reactions using immunoprecipitated Jak1 or Jak2 (Fig. 1d). IFN- γ induced an increase in the kinase activity of both Jak1 and Jak2, whereas IFN- α stimulated the kinase activity only of Jak1, correlating with the tyrosine phosphorylation data of the Jak proteins. Phosphoryla-

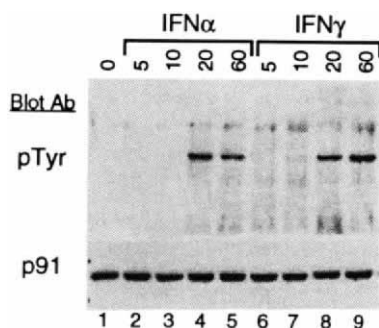
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tion of Jak1 in response to IFNs has been observed recently by others¹⁵.

As both IFN- α and IFN- γ stimulate tyrosine phosphorylation of ISGF3 α p91 (refs 3, 4), the kinetics of p91 phosphorylation and Jak1 and Jak2 autophosphorylation were compared (Fig. 2). IFN- α -induced phosphorylation of p91 was maximal within 20 min and diminished after 1 h of stimulation (Fig. 2, lanes 2–5); IFN- γ -stimulated phosphorylation of p91 increased for at least 1 h (lanes 6–9). Abundance of p91 remained unchanged (Fig. 2, lower panel). These kinetics correlate with the kinetics of Jak1 and Jak2 phosphorylation in response to IFNs (see Fig. 1). They also correlate with stimulation of p91 DNA-binding activity, which is more rapid in response to IFN- α than to IFN- γ (ref. 4).

These data, combined with other recent reports^{13–16}, implicate two kinases in each IFN pathway: Tyk2 and Jak1 respond to IFN- α , and Jak1 and Jak2 respond to IFN- γ . Two possible models can be envisaged: the activated kinases may phosphorylate different substrates or the kinases may interact with each other to stimulate gene expression. For instance, phosphorylation of p91, which is common to both IFNs, might be mediated by Jak1, whereas Tyk2 would phosphorylate p113 specifically in response to IFN- α . In response to IFN- γ , Jak2 might be required to activate an unidentified moiety necessary for p91 phosphorylation, as Jak2-deficient cells fail to activate p91 in response to IFN- γ (ref. 14). In an alternative model, the kinases might act together as heterodimers or sequentially to phosphorylate p91. To investigate these possibilities, we studied cells transfected with mammalian expression vectors directing the synthesis of either Jak1 or Jak2. Overexpression of Jak kinases resulted in ligand-independent tyrosine autophosphorylation (Fig. 3a). Jak kinases displayed restricted substrate specificity compared with other kinases. The only prominent tyrosine phosphorylated protein detected in cell lysates comigrated with the Jak kinases themselves (Fig. 3a, 1, 2). In contrast, overexpression of Src (lane 4) or Lck kinases (not shown) resulted in phosphorylation of multiple cellular proteins. Overexpression of Jak1 or Jak2 also activated p91 DNA-binding activity (Fig. 3b, lanes 2, 3). In contrast, cells transfected with vector alone (Fig. 3b, lane 1), or with activated c-Src bearing a carboxyl-terminal Tyr-to-Phe mutation (Fig. 3b, lane 4), failed to display significant specific DNA-binding activity. Normal cellular Src, Lck or activated Lck also failed to activate p91 (not shown). Antibody to p91 (Fig. 3c, lanes 1, 2), but not antibody against ISGF3 48K subunit (lanes 3, 4), produced a supershifted band, indicating that both the Jak1- and Jak2-induced DNA-binding activities involved p91. Biological consequences of p91 activation by overexpressed Jak kinases was tested in transcription assays. COS cells cotransfected with a luciferase reporter construct containing a p91-dependent promoter⁵ and either Jak1 or Jak2 led to 3.5- and 3.2-fold transcriptional induction, respectively. In contrast, expression of c-Src slightly suppressed transcription of the reporter.

FIG. 2 Kinetics of IFN- α and IFN- γ -induced tyrosine phosphorylation of p91. Serum-starved 3T3 cells were untreated or treated for the indicated times with IFN- α or IFN- γ as described for Fig. 1. Cell lysates were immunoprecipitated with anti-p91 serum¹ and blotted with anti-phosphotyrosine (upper panel) or anti-p91 antibodies (lower panel).



Because overexpression of either Jak kinase activated p91 DNA-binding activity, we considered whether kinase cross-activation indicative of a signalling cascade might be involved. Jak1 and Jak2 were immunoprecipitated from transfected cells and tyrosine phosphorylation was analysed as an indicator of their activation (Fig. 4). As expected, immunoprecipitated Jak1 from Jak1 transfectants was heavily phosphorylated on tyrosine residues (Fig. 4a, lane 3); also, weak stimulation of endogenous Jak1 phosphorylation was detected in cells overexpressing Jak2 (lane 2). Similarly, endogenous Jak2 immunoprecipitated from Jak1-transfected cells (Fig. 4a, lane 5) displayed significant tyrosine phosphorylation. This Jak1-induced tyrosine phosphorylation of endogenous Jak2 was not due to co-immunoprecipitation of transfected Jak1. Reprobing of immunoblots with anti-Jak1 antibodies demonstrated the absence of contaminating Jak1 protein in Jak2 immunoprecipitates (Fig. 4a, middle panel). Additionally, the levels of Jak1 and Jak2 expression after transfections were similar (Fig. 4b). These results suggest that Jak1 and Jak2 are capable of both trans- and autophosphorylation, with Jak1 being a more effective activator of Jak2. Transphosphorylation was also observed *in vitro* (Fig. 4c). Overexpressed Jak1 and Jak2 specifically phosphorylated a peptide containing a Jak2 putative phosphorylation site but failed to phosphorylate a control tyrosine-containing synthetic peptide.

Previous genetic analysis has demonstrated a requirement for Tyk2 only in response to IFN- α and for Jak2 only in response to IFN- γ . We have found that Jak2 is indeed activated specifically in response to IFN- γ whereas Jak1 seems to be shared by both pathways. As phosphorylation of ISGF3 p91 is a common step in response to both IFNs, Jak1 might be the p91 kinase. In support of this notion, overexpression of Jak1 leads to ligand-independent activation of p91. However, Jak1 expression also activated Jak2, which in turn was capable of activating p91 on

FIG. 3 Overexpression of Jak1 and Jak2 induces p91 DNA-binding activity. a, Overexpression of Jak kinases leads to tyrosine autophosphorylation. Mammalian expression vectors, directing the synthesis of Jak1, Jak2 or Src(Y-F), were used in transient transfections, and total cell lysates were analysed by anti-phosphotyrosine immunoblotting. b, Jak1 and Jak2 overexpression stimulates p91 DNA-binding activity. p91 DNA-binding activity in nuclear extracts was assayed by electrophoretic band shift from cells transfected as indicated. c, DNA-binding activity from Jak1- and Jak2-transfected cells was abrogated by addition of antibody to p91 (lanes 1, 2) but not to p48 (lanes 3, 4). METHODS. Murine Jak1, Jak2, Lck²¹, activated Lck (Y505F)²² and chicken c-Src²³ or activated c-Src (Y527F)^{24–27} were cloned into a cytomegalovirus-based vector. Expression constructs were transfected into human embryonic kidney 293 cells by calcium phosphate precipitation (a) or into COS-1 cells by DEAE-dextran (b, c) by standard methods. Thirty-six hours after transfection, cells were lysed and total cell lysates (a) were separated on SDS-gels and subjected to anti-phosphotyrosine immunoblotting as for Fig. 1. DNA-binding activity in nuclear extracts (b, c) was assayed by electrophoretic band shift as described previously^{4,28}.

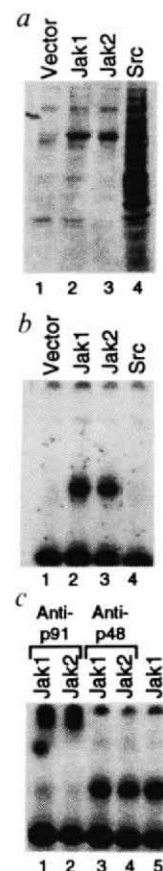
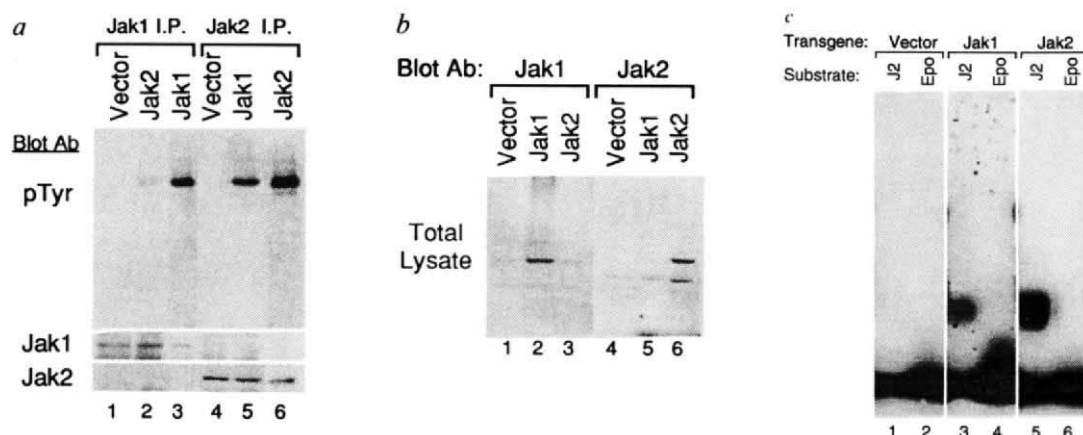


FIG. 4 Transactivation of Jak tyrosine kinases. COS-1 cells were transfected either with vector alone or with Jak1 or Jak2 expression vectors as for Fig. 3b. Cell lysates were immunoprecipitated with anti-Jak1 or anti-Jak2 and immunoblotted with anti-phosphotyrosine antibodies (upper panel). The filters were stripped, reprobed with anti-Jak2 (lower panel), stripped again and probed with anti-Jak1 antisera (middle panel). The amount of immunoprecipitated protein loaded in lanes 3 and 6 was decreased to correspond to endogenous protein levels. *b*, The expression levels of Jak kinases after transfection were monitored from total cell lysates by immunoblotting with anti-Jak1 and anti-Jak2, as indicated. *c*, Jak1 (lanes 1–4) and Jak2 (lanes 5–6) were immunoprecipitated from cells transfected as indicated. *In vitro* kinase assays were



performed in the presence of synthetic peptides (1 mg ml^{-1}) corresponding to the putative tyrosine phosphorylation sites of Jak2 (VLPQDKKEYKVKPEGE, lanes 1, 3, 5) or erythropoietin receptor (GDSSDGPYPYENSLVA, lanes 2, 4, 6). Products of the kinase reactions were separated on 20% SDS-PAGE and autoradiographed.

overexpression. These data suggest that Jak1 and Jak2 act together to stimulate p91 DNA binding in response to IFN- γ . This implies a precise modulation of Jak kinases dependent on the activating receptor.

Jak2 is also activated in response to interleukin-3 and erythropoietin^{17,18}; however, these ligands do not activate p91 phosphorylation (ref. 15, and J.N.I., unpublished data). Rather, they stimulate a distinct DNA-binding protein (O.S. and D.E.L., unpublished data). It has been proposed that p91 associates directly with the epidermal growth factor receptor¹⁹, allowing it to be activated in response to growth factor stimulation^{4,20}. Therefore, target specificity may be determined by recruitment of distinct transcription factors and particular Jak-family kinases to a ligand-activated receptor. □

Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4

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THE human Ras-related nuclear protein Ran/TC4 (refs 1–4) is the prototype of a well conserved family of GTPases that can regulate both cell-cycle progression^{5–8} and messenger RNA transport⁹. Ran has been proposed to undergo tightly controlled cycles of GTP binding and hydrolysis, to operate as a GTPase switch^{10,11} whose GTP- and GDP-bound forms interact differentially with regulators and effectors. One known regulator, the protein RCC1 (refs 12, 13), interacts with Ran to catalyse guanine nucleotide exchange, and both RCC1 and Ran are components of an intrinsic checkpoint control that prevents the premature initiation of mitosis. To test and extend the GTPase-switch model, we searched for a Ran-specific GTPase-activating protein (GAP), and for putative effectors (proteins that interact specifically with Ran/TC4-GTP). We report here the identification of a Ran GAP and its use to characterize the GTP-hydrolysing properties of mutant Ran proteins, and the identification and cloning of a binding protein specific for Ran/TC4-GTP.

A wild-type Ran/TC4 complementary DNA¹ and a cDNA mutated at codons 19 and 69 (analogous to activating mutations of H-ras codons 12 and 61 (refs 2, 14)) were used to generate recombinant Ran/TC4 proteins (Fig. 1a). Wild-type and mutant proteins both bound GTP, but neither catalysed appreciable hydrolysis of the nucleotide over a 60-min incubation (Fig. 1b).

We then used these GTP-charged proteins as substrates to search for a GAP activity. When wild-type Ran/TC4-GTP was incubated in the presence of a HeLa cell S-100 extract, hydrolysis of the bound GTP proceeded briskly (Fig. 1b). When this HeLa-S100 extract was subjected to gel filtration, essentially all GAP

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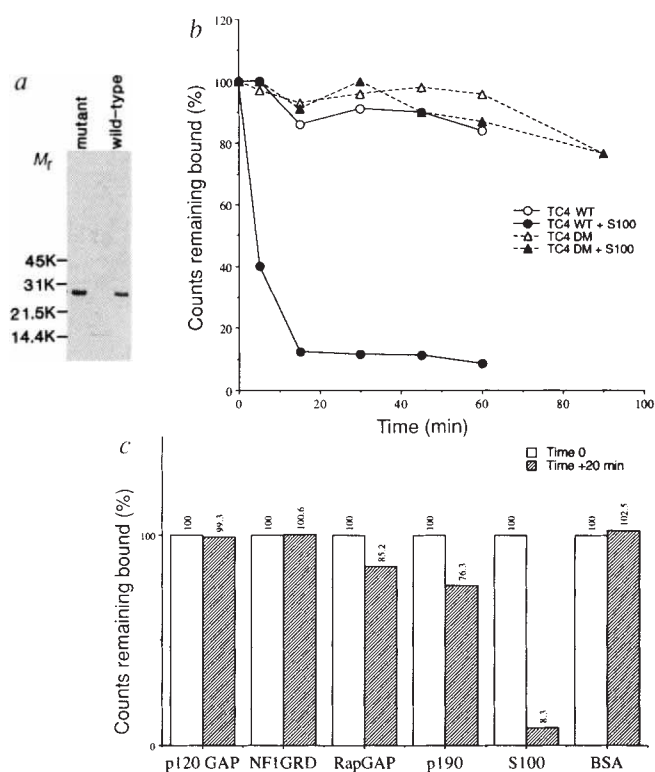
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FIG. 1 A Ran-specific GAP activity. *a*, Characterizations of purified recombinant Ran/TC4 proteins. Wild-type Ran/TC4 (1 μ g) and 2 μ g of GTPase-defective mutant Ran/TC4 were fractionated on a 15% SDS-PAGE gel and stained with Coomassie blue. *b*, Hydrolysis of GTP bound to wild-type, but not mutant, Ran/TC4 in the presence of a HeLa S-100 extract. Recombinant wild-type or mutant Ran/TC4 proteins charged with [γ - 32 P]GTP were incubated in the presence or absence of S-100 extract, and assayed for GTPase activity by filter binding. *c*, Wild-type Ran/TC4 GTPase activity is not stimulated significantly by the purified Ras GAPs p120 and NF1GRD, or by a Rho (p190) or Rap GAP. (p190 was from R. Weinberg; all others were from F. McCormick.) HeLa S-100 and BSA were included as positive and negative controls, respectively. METHODS. Recombinant Ran/TC4: wild-type and mutant Ran/TC4 cDNAs² were cloned into pET-9C (ref. 21), expressed in *Escherichia coli* BL21(DE3), and recombinant proteins purified as in ref. 4, after lysis of bacteria in a French press. GTPase assay: Ran/TC4 proteins (1 μ M) charged with [γ - 32 P]GTP (0.5 μ M, 1,000 Ci mmol⁻¹; NEN) by incubation for 20 min at 25° in 50 mM Tris pH 7.4, 40 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 1 mg ml⁻¹ BSA, were adjusted to 10 mM MgCl₂, 5 mM NaCl, and incubated in the presence or absence of GAPs (~1 μ g of each purified GAP, or 1 μ g HeLa S-100 protein per 40 μ l reaction mixture) at 25 °C. Reactions were terminated by addition of 1 ml ice-cold 50 mM Tris pH 7.4, 20 mM MgCl₂, 1 mM DTT, 2 mg ml⁻¹ BSA, washed through nitrocellulose filters and subjected to scintillation counting. Ran/TC4 proteins charged with [α - 32 P]GTP (instead of [γ - 32 P]GTP) and assayed for GAP activity as described here, exhibited no loss of bound radioactivity (data not shown).

FIG. 2 Ran/TC4-GTP, but not Ran/TC4-GDP, binds to specific proteins on blots of mammalian, *Xenopus*, and fission yeast extracts. *a*, Autoradiograph of a blot of tsBN2 (hamster cell line) protein (50 μ g per lane) probed with Ran/TC4-[γ - 32 P]GTP complex. Lanes include total protein (lysate) or S-100 and P-100 fractions. *b*, Competition autoradiograph of a blot of tsBN2 S-100 protein (20 μ g per lane), probed with Ran/TC4-[γ - 32 P]GTP in the presence of excess unlabelled Ran/TC4-GTP or Ran/TC4-GDP. *c*, Autoradiograph of a blot of *Xenopus* cytosolic, *Schizosaccharomyces pombe* total, and HeLa S-100 protein (20 μ g per lane each) probed with Ran/TC4-[γ - 32 P]GTP complex. METHODS. S-100 (supernatant) and P-100 (pellet) fractions were prepared by sonicating cells on ice in 25 mM HEPES pH 7, 150 mM NaCl, 5 mM MgCl₂, 3 mM DTT, 100 U ml⁻¹ aprotinin, 10 μ g ml⁻¹ leupeptin, followed by centrifugation at 100,000g for 1 h at 4 °C. Western blots of proteins fractionated by SDS-PAGE on 12% gels, transferred to nitrocellulose membranes in 25 mM Tris, 192 mM glycine, 15% methanol, pH 8.3, were renatured by a 48 h incubation at 4 °C in 50 mM HEPES pH 7, 100 mM potassium acetate, 5 mM magnesium acetate, 3 mM DTT, 0.3% Tween-20, 1% BSA²². Blots were preincubated in renaturation buffer containing 0.1% Tween-20 and 250 μ M GTP for 30 min at 25 °C, and then in the same buffer containing 0.5 μ g ml⁻¹ Ran/TC4-[γ - 32 P]GTP (30 Ci mmol⁻¹) for 45 min at 4 °C. Filters were washed 5 times in preincubation buffer without GTP and autoradiographed. The Ran/TC4-[γ - 32 P]GTP probe was an equimolar mixture of wild-type and GTPase-defective mutant proteins (8 μ M total) charged with [γ - 32 P]GTP (8 μ M). For the competition experiments, the probing buffer contained either 10-fold excess unlabelled Ran/TC4-GTP or 10-fold excess unlabelled Ran/TC4-GDP.

activity was recovered as a single broad peak of M_r >100K (data not shown). The GAP activity defined in this assay may be Ran-specific: incubation of wild-type Ran/TC4-GTP with purified Ras GAPs (p120 and NF-1), a Rap-1 GAP, and Rho GAP p190, stimulated no GTP hydrolysis above background levels (Fig. 1c). A 65K protein with similar GAP prop-



erties, present as a homodimer in HeLa cell extracts, has been purified¹⁵.

Mutant Ran/TC4-GTP showed no GTPase activity in the presence of HeLa S-100 extract, confirming that mutations in Ran/TC4 codons 19 and 69 block the protein's GTPase activity. As the intrinsic GTP hydrolysis rate of wild-type Ran/TC4 is very low, the mutant defect is manifested *in vitro* as its insensitivity to stimulation by GAP. These results establish that Ran/TC4 protein has a GAP-dependent GTPase activity which is abolished by mutations known to affect its function *in vivo*².

To search for Ran/TC4 interacting proteins, we probed renatured blots of human (HeLa) and hamster (tsBN2) cell extracts with Ran/TC4-[γ - 32 P]GTP. We detected a polypeptide with an apparent M_r of 27K. The results for tsBN2 cells are shown in Fig. 2a. No proteins were detected in blots probed with Ran/TC4-GDP labelled to 10 times the specific activity of the GTP used in these experiments (data not shown). Binding by Ran/TC4-[γ - 32 P]GTP was inhibited in the presence of excess Ran/TC4-GTP but not excess Ran/TC4-GDP (Fig. 2b). Immunoblotting with Ran/TC4 anti-peptide antibody² demonstrated that the 27K protein was distinct from Ran/TC4, and gel filtration resolved it from Ran/TC4 GAP activity (data not shown). The protein corresponding to the 27K band has been named Ran-binding protein-1 (RanBP1).

To clone RanBP1, we probed an expression library containing cDNAs from 16-day mouse embryos with Ran/TC4-[α - 32 P]GTP, and plaque-purified five clones (Fig. 3a). All five were of different sizes, and thus appear to be independent, but the rescued cDNAs all contained the same open reading frame (Fig. 4); one lacked the 60 amino-terminal codons. The nucleotide sequence of the open reading frame is 98% identical to that of the mouse HTF9A cDNA (ref. 16). HTF9A was discovered in the course of a search for bidirectional promoters; its function is unknown. The protein is highly charged (40% Glu+Asp+Lys+Arg) and acidic, with a predicted M_r of 23,600. It contains no clear diagnostic catalytic domains, but does contain two regions close to the consensus sequence for a leucine zipper, one close to that for a helix-turn-helix, and one close to that for an RNA-binding motif (Fig. 4).

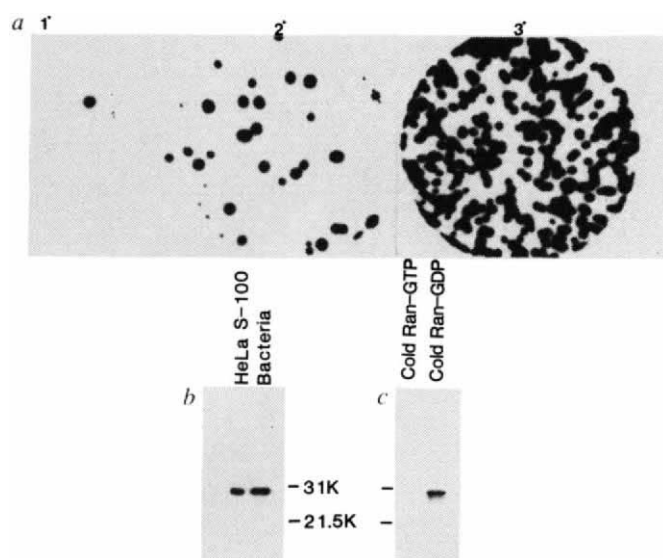


FIG. 3 Use of Ran/TC4-GTP as a probe for the cloning of RanBP1, a putative Ran target. **a**, cDNA library screening with a Ran/TC4- $[\alpha^{32}\text{P}]\text{GTP}$ probe. Autoradiograms of the primary, secondary and tertiary filter lifts of one of the positive plaques are shown. **b**, The cloned Ran/TC4 binding protein is indistinguishable from the Ran/TC4 binding protein identified in mammalian cell extracts. **c**, Recombinant RanBP1 binds Ran/TC4-GTP specifically; competition autoradiograph of a blot of partially purified recombinant RanBP1 probed with Ran/TC4- $[\gamma^{32}\text{P}]\text{GTP}$ in the presence of excess unlabelled Ran/TC4-GTP or Ran/TC4-GDP.

METHODS. Library screen: screenings were done essentially as described in ref. 23. Filter lifts were washed in 10 mM Tris, pH 8, 150 mM NaCl, 0.05% Triton X-100 (TBSX), at room temperature, blocked for 4 h at 4 °C in 20 mM HEPES, pH 7.5, 25 mM potassium acetate, 5 mM MgCl_2 , 5 mM DTT, 50 μM GTP, 50 μM GDP, 4% non-fat dry milk, 0.05% Tween-20, and probed for 2 h at 4 °C in 50 ml of the same buffer plus 10 mM magnesium acetate and 10^6 c.p.m. ml^{-1} (125 ng ml^{-1}) Ran/TC4- $[\alpha^{32}\text{P}]\text{GTP}$. Filters were washed 5 times in TBSX, 5 mM magnesium acetate. In competition experiments using the protocol described for Fig. 2b, binding to tertiary plaques was specific for Ran/TC4-GTP (data not shown). SDS-PAGE analysis: 10 μg of HeLa S-100 extract proteins and 0.1 μg of *E. coli* BL21(DE3) extract expressing RanBP1 were electrophoresed, blotted, and probed with Ran/TC4- $[\alpha^{32}\text{P}]\text{GTP}$ as described in the legend of Fig. 2. The anomalously slow mobility of RanBP1 on SDS-PAGE may be due to its low isoelectric point ($\text{pI}=5.2$). Competition experiments: 0.1 μg each of RanBP1-expressing bacterial extracts was electrophoresed, blotted and probed with Ran/TC4- $[\gamma^{32}\text{P}]\text{GTP}$ as for Fig. 2b.

The 609-base-pair (bp) open reading frame from one of the cDNAs was used to generate recombinant protein. When analysed by SDS-polyacrylamide gel electrophoresis, purified recombinant RanBP1 migrated at the same rate as RanBP1 from a HeLa S-100 extract (Fig. 3b). Purified recombinant RanBP1 had no Ran/TC4-specific GAP activity (data not shown). Because Ran/TC4-GTP but not Ran/TC4-GDP interacts with RanBP1 (Figs 2b and 3c) and because RanBP1 has negligible GAP activity, RanBP1 may be a Ran/TC4 effector.

Ran proteins are highly conserved, leading us to expect that effector domains interacting with them would also be well conserved. Accordingly, a cytosolic extract of *Xenopus* oocytes and a total extract of fission yeast were subjected to SDS-PAGE, blotted, renatured, and probed with Ran/TC4- $[\gamma^{32}\text{P}]\text{GTP}$. Single Ran/TC4-interacting proteins were identified in each extract (Fig. 2c).

Ran and RCC1, the Ran-specific guanine-nucleotide-exchange protein, are implicated in diverse cellular functions¹⁷. In fission yeast and mammals, alterations in either protein perturb a checkpoint between the completion of DNA synthesis and the onset of mitosis (M.R., unpublished results, and refs

cDNA	1	CCCCATGGCGCCGCCAAGGACAGTCACGAGGACCATTGATCTTCCACAGAGAATGCGAG	60
protein		1 M A A A K D S H e D H D T S T E N A D 19	
	61	ATGAGTCCAAcCaagACCCCCAGTTCGAGCAATAGTTTCTGTTCCGAGCAAGAAATTA	120
	20	E S n h d P Q F E P I V S V P E Q E I K 39	
	121	AAACGCTGGAGGAAGATGAAGAGGAACTTTTTAAGATGCGTCAAGAGCTGTTCCGGTTTG	180
	40	T L* E E E D E E E L* F K M R A K L* F R F A 59	
	181	CTTCAGAGAATGACCTCCCAAGATGAAGGAGGagGcaCTGGAGATGTCAAGCTTCTGA	240
	60	S E N D L P E W K E r g t G D V K L L K 79	
	241	AGCACAAAGGAGAAAGGACCATCCGCTTCTTATGAGGAGGACAAAACCTTGAAGATAT	300
	80	H K E K G T I R L L* M R R D K T L* K I C 99	
	301	GCGCAACCACTATATTACCAATGATGGAGCTGAAGCCGAATGCTGGCAGTGACCGAG	360
	100	A N H Y I T P M M E L* K P N A G S D R A 119	
	361	CCTGGGTCTGGAATACCCACgCCGACTTGTCTGACGAGTGCCTCCCAAGCCTGAGCTGCTG	420
	120	W V W N T H a d F A D E C K P E L L L A 139	
	421	CCATCCGCTTCTTAAATGCTGAGAATGCAAAAAGTTTGAAGAAATGCA	480
	140	I R F L N A E N A Q K F K T K F E E C R 159	
	481	GGAAGAAATGAAGAGAGAGAAAGAAAGGACAGGCAAAAATGATAATGCCGAAAAG	540
	160	K E I E E R E K K G P G K N D N A E K V 179	
	541	TGGCCGAGAAGCTGGAAGCCCTTTCAGTGAGGAGGCGAGAGAGGAGCTGAAGAGAAGT	600
	180	A E K L E A L S V R E A R E E A E E K S 199	
	601	CTGAGGAGAAACATGAATCACTCTGCTTTTCTTCTTCT 641	
	200	E E K Q ter 203	

FIG. 4 RanBP1 nucleotide and predicted amino-acid sequences. The complete nucleotide sequence of a single full-length clone (GenBank accession number L25255) was determined on both strands using 'Sequenase' T7 DNA polymerase (US Biochemicals). Differences with the HTF-9A nucleotide and predicted amino-acid sequences (ref. 16; GenBank accession number X56045) are shown in lower-case letters (single-letter code). '|--->' marks the N-terminal end of the truncated clone that showed Ran/TC4-GTP binding activity. In the amino-acid sequence, a potential RNA-binding site consensus is underlined; asterisks indicate leucines in two potential zipper regions, and a potential helix-turn-helix motif is shown in bold.

5, 6, 8, 12, 17). In budding yeast and mammals, such alterations can perturb mRNA export from the nucleus^{9,17,19}. In one *in vitro* model for protein import into nuclei, purified recombinant Ran/TC4 protein substitutes for a 25K *Xenopus* protein fraction²⁰. These functions may be interconnected; Ran and RCC1 may affect cell-cycle progression as part of their overall role in nuclear transport, by regulating the traffic in mRNAs and/or proteins required for the onset of mitosis. RanBP1, which interacts specifically with GTP-charged Ran and has a putative RNA-binding site, is a plausible intermediary in this process. □

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Replication structure of the human β -globin gene domain

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THE animal cell genome is organized into a series of replicons with an average size of 50–300 kilobases¹; each of these units is characterized by its own origin of replication which serves as the point of initiation for DNA synthesis. In animal viruses, origin usage can be regulated by *cis*-acting elements², and in some cases, replication may be cell-type specific³. Little is known, however, about the organization and control of endogenous tissue-specific gene replication. To understand this process, we have used a replication direction assay to examine DNA fragments covering more than 200 kilobases of the human β -like globin domain, and have identified a single bidirectional origin located upstream of the β -globin itself. This locus is used to initiate DNA synthesis in expressing cells, where the globin domain replicates early, and in non-expressing cells, which are characterized by late replication of the same region^{4,5}. Deletion of this origin sequence, as occurs in the haemoglobin Lepore syndrome⁶, cancels bidirectional DNA synthesis at this site and leads to a striking reversal of replication direction upstream to the locus. This represents the first genetic proof of the existence of specific, discrete origins of replication in animal cells.

We have developed a general approach for detecting bidirectional origins by assaying replication directions of individual genomic DNA fragments and then identifying sites of tail-to-tail DNA synthesis⁷. In this procedure, bromodeoxyuridine (BrdU)-labelled DNA is isolated from emetine-treated cells in order to separate the newly synthesized leading-strand DNA. This BrdU DNA is hybridized with strand-specific probes, and it is the unequal hybridization pattern which then determines the direction of replication. An example of this approach for the 900-base-pair (bp) *Hind*III/*Eco*RI fragment located about 25 kb downstream of the β -globin gene is shown in Fig. 1. In this case, the BrdU-labelled leading-strand DNA from K562 cells hybridizes preferentially to the minus-strand probe, indicating that DNA replication at this site proceeds in the downstream direction. The general efficacy of this technique has been confirmed by using it to detect a bidirectional origin downstream of the Chinese hamster dihydrofolate reductase gene⁷ in the region previously identified by a variety of different molecular approaches^{8–11}.

To gain some insight into the chromosomal replicon structure of tissue-specific genes, we carried out an evaluation of the human β -globin domain. A complete analysis of replication directions using a large number of probes distributed over this entire region reveals a clearcut pattern of bidirectional replication radiating outwards from an origin locus slightly 5' to the β -globin gene (Fig. 2). This pattern of replication directions is observed in K562, which contains an active globin domain, but results are similar when BrdU DNA is used from fibroblasts and lymphoblasts, cells that do not express any of these globin genes. This suggests that the β -globin origin is a constitutive feature of the domain whose basic activity is not subject to developmental control. The issue has not yet been resolved of whether animal cell DNA synthesis initiates at small discrete loci¹⁰ or within a diffuse origin region¹¹. The observation that probes H and I replicate in opposite directions, however, indicates that the β -globin replication origin must be located within a small, well-defined 2-kilobase (kb) fragment, and it is worth noting that this very region contains a unique sequence motif that is a common element in other eukaryotic origins¹².

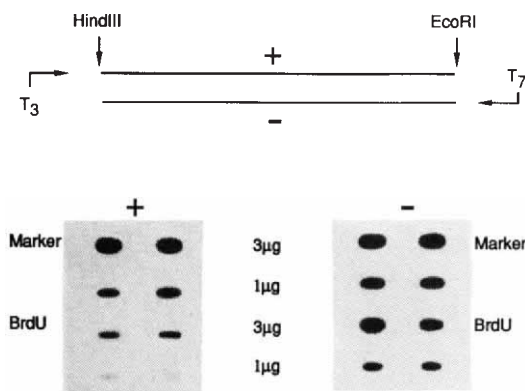
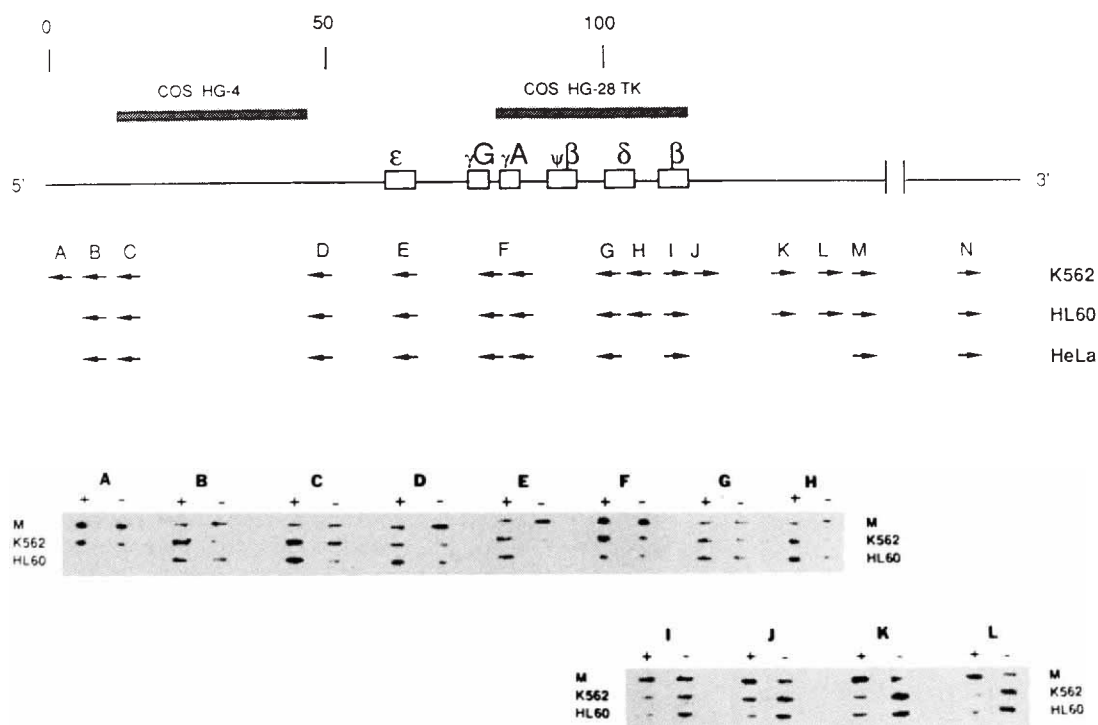


FIG. 1 Replication direction assay. The 900-bp *Hind*III/*Eco*RI fragment located ~25 kb downstream of the β -globin gene was cloned into the polylinker of the pBS vector as shown. The plus strand (+) riboprobe synthesized by T3 RNA polymerase or the minus strand (–) probe synthesized by T7 RNA polymerase were hybridized to identical filters containing ~1 and 3 μ g BrdU DNA or total marker DNA from K562. All samples were assayed in duplicate. Marker DNA is included in every experiment to correct for the differences in hybridization efficiencies of the two complementary riboprobes. The plus probe hybridizes poorly to BrdU DNA but the minus probe gives a strong signal. Thus in this region, the plus DNA represents the leading strand, and we can conclude that this fragment replicates to the right.

METHODS. Logarithmic (1×10^8) cells were grown in medium containing 10 μ M BrdU, 10 μ M 5-fluorodeoxyuridine and 2 mM emetine-HCl for 24 h. Cells were treated with proteinase K at 55 °C overnight in 10 mM EDTA, 1% (v/v) Sarkosyl, and lysates were extracted with phenol and chloroform-isoamyl alcohol (24:1 v/v), precipitated in 67% (v/v) ethanol at –20 °C, redissolved in 10 mM Tris-HCl, pH 7.8, 1 mM EDTA, and reduced to an average size of 300–500 bp by either sonication or DNase I treatment¹⁸. In some cases, DNA was isolated from micrococcal-nuclease-treated nuclei⁷. DNA was then dissolved in 4.5 ml 0.1 M NaOH, 10 mM EDTA, adjusted to a density of 1.749 g cm^{–3} by adding Cs₂SO₄, and centrifuged to equilibrium at 54,000 r.p.m. in a VTi-65 vertical rotor for 24 h at 20 °C. Gradient fractions (0.2 ml) containing newly replicating DNA were identified by immunoblotting with an anti-BrdU antibody (Becton Dickinson) in conjunction with an alkaline phosphatase detection system (Vector) and distinguished from the light DNA peak (labelled with a ¹⁴C-dT marker)⁷, and then pooled and dialysed against 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl. Previously it was assumed that micrococcal nuclease treatment of nuclei was necessary, together with emetine to take advantage of preferential nucleosome segregation to the leading strand^{19,20}, but analysis of Okazaki fragments and electron microscopy have shown that emetine alone inhibits the synthesis of the lagging strand. This provides a good mechanistic basis for measuring replication directions and enables the assay to be done without nuclease treatment¹⁸. Strand-specific RNA probes of high specific activity were made using T3 and T7 polymerases⁷.

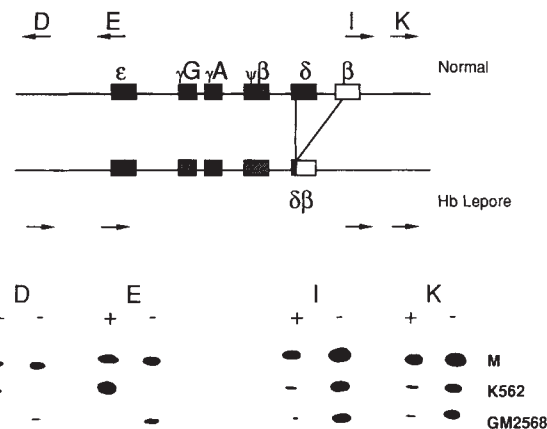
What happens to DNA replication when a chromosomal origin element is removed? To answer this question, we used cell line GM2568 derived from a homozygous Hb Lepore patient carrying a small deletion between the δ and β genes⁶ which includes the presumptive origin sequence. Analysis of DNA replication directions in these cells (Fig. 3) shows that fragments 3' to the β -globin gene still replicate in the downstream direction. Strikingly, however, the DNA located 5' to this gene has switched from its wild-type pattern and now replicates in the downstream direction as well. This experiment provides straightforward genetic evidence for the existence of a functional chromosomal origin near the β gene. We assume that in the mutant cell line, replication of the globin domain is directed by an alternative origin located in a 5' position either at a cryptic site or from the adjacent upstream replication unit. This is similar to the situation in yeast *Saccharomyces cerevisiae*, in which removal of endogenous origin sequences from one large section of chromosome III causes this entire stretch of DNA to be passively

FIG. 2 Replication direction analysis of the β -globin domain. Map (kb scale) of human β -globin domain is shown with the locations of the gene sequences and the approximate positions of the cosmid clones cos HG-4 and cos HG-28TK. Fragments of DNA from this domain were cloned into pBS or Bluescript and then used to make strand-specific riboprobes. Filters were prepared with slots containing $\sim 1 \mu\text{g}$ BrdU DNA from both the K562 and the HL60 cell lines, together with $1 \mu\text{g}$ marker DNA, and identical filters were then hybridized to the plus and minus strand riboprobes. Hybridization to the marker DNA served to normalize for differences in probe efficiency. Several concentrations of BrdU and marker DNA were assayed, but only one concentration is shown. Results for probe M are presented in Fig. 1 and those for probe N are not shown. The directions of replication for all probes are summarized schematically for K562, HL60 and HeLa (data not shown) cells. We have also verified that this entire region replicates early in the cell cycle in K562 and late in HL60 and HeLa¹³. For all fragments, experiments were repeated several times using different preparations of BrdU DNA. Autoradiograms were scanned and the data used to calculate the bias number of one strand compared with the other after normalization for marker hybridization efficiency. The bias number averages for both cell types in this experiment are as follows: to the left, A(4), B(11), C(10), D(6), E(21), F(5), G(4), H(8); to the right, I(5), J(13), K(9), L(12). Control samples from HeLa cells not treated with emetine showed a bias number of 1.2 for probe C and 1.0 for probe E. The restriction fragment probes are described below. Where relevant, the site numbers in parentheses refer to the position of the fragments in genebank sequence HUMHBB.PR, which begins 19,500 bp upstream of the ϵ gene. All probes contain unique DNA sequences, as confirmed by Southern blot hybridization; fragments with known repeats were not used. 'A' is a 0.5-kb *EcoRI*/*NcoI* fragment subcloned from a plasmid containing the 5' $\gamma\delta\beta$ -thal 1 breakpoint²¹ (from E. F. Vanin). This



DNA is part of a normal 0.9-kb *EcoRI* fragment located at position +5 kb on the map²². The 'B' (1.2-kb *EcoRI*/*PstI*) and 'C' (1.0-kb *XbaI*/*EcoRI*) fragments were subcloned from the 5' and 3' ends of the 4.1-kb *EcoRI* fragment (from F. Grosfeld) located at position +10 kb on the map. 'D' is a 1.4-kb *BamHI*/*HindIII* fragment (3,877–5,172) (from O. Smithies); 'E', 1.3-kb *BamHI*/*EcoRI* fragment (19,977–21,251) of the ϵ gene; 'F', 0.6-kb *EcoRI*/*HindIII* fragment which appears at the 3' end of both the γG (35,920–36,482) and γA (40,836–41,382) genes; and 'G', 1-kb *IVS2\delta* *BamHI*/*EcoRI* fragment (55,266–56,231) (fragments E–G from A. Oppenheim); 'H', 0.7-kb *BamHI*/*BglII* fragment (59,933–60,629) located 3' to the δ gene; 'I', 0.9-kb *BamHI*/*EcoRI* *IVS2\beta* fragment (62,665–63,582); and 'J', 0.9-kb *AccI*/*HincII* fragment (65,917–66,782) located 3' to the β gene; H–J were all subcloned from plasmid pH β 9 (all from T. Maniatis), K, L and M, the 1.1-kb *RK28* *EcoRI*/*SaII* fragment, the 1.1-kb *RK29* *BglII*/*EcoRI* fragment and the 0.9-kb *900HE* *HindIII*/*EcoRI* fragment, respectively, are all ~ 20 –30 kb downstream of the β gene²³ (all from C. L. Schildkraut). 'N' is a 1.1-kb *SstI*/*BamHI* fragment from the 3' breakpoint region of HPFH-1 and HPFH-2 (ref. 24), obtained from M. Groudine and E. Epner. This probe is >100 kb downstream from the β gene⁵, and may not be part of the same replicon.

FIG. 3 Replication direction in the Hb Lepore cells. DNA in the Hb Lepore syndrome is characterized (see map) by an 8-kb deletion that generates a δ - β fusion gene⁶. The GM2568 cell line (Camden tissue culture repository) is homozygous for this deletion²⁵. Plus-strand and minus-strand DNA from each of the probes shown in the map were used to assay BrdU DNA from K562 and GM2568 cells. Marker DNA was included on each filter to standardize the hybridization efficiencies. The directions of replication (arrows) are shown graphically for both the normal domain (top) and the Hb Lepore domain (bottom). In addition to the probes shown, there was a reversal of direction in GM2568 for the 500H *HinfI* fragment²³ located about 3 kb upstream to the δ gene (data not shown).



replicated from a distant origin (C. Newlon, personal communication).

As the globin replicon seems to be quite large, sequences nearer the origin should replicate considerably earlier than those at the periphery in each individual cell. This prediction can actually be verified using fluorescence *in situ* hybridization to interphase nuclei. Any specific DNA locus that has already replicated shows a double hybridization signal in that cell, whereas the

Cell type	Double HG-28TK	Double HG-4
	Single HG-4	Single HG-28TK
K562	23% (31/135)	6% (9/146)
HL60	23% (53/226)	6% (17/276)
GM2568	5% (12/232)	19% (38/205)

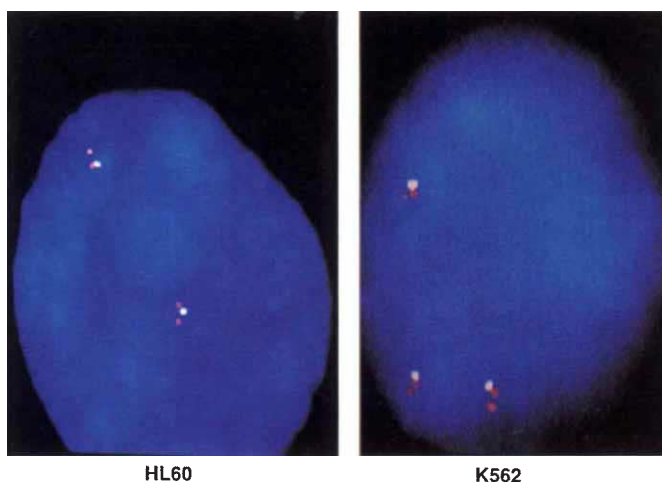


FIG. 4 *In situ* hybridization analysis of the β -globin domain. Nuclei from elutriated fractions of K562, HL60 and GM2568 were hybridized simultaneously with digoxigenin-labelled cos HG-28TK and biotin-labelled cos HG-4. Biotin was detected with avidin-fluorescein isothiocyanate (FITC) (yellow), and digoxigenin (DIG) with anti-DIG-rhodamine isothiocyanate. For each cell type, nuclei were assayed twice. In the first survey, chromosomes with single FITC (HG-4) dots were identified and the status of the second probe (HG-28TK) at the same locus was then determined. This assay measures the percentage of cells in which the replication fork has already passed the HG-28TK region, but has not yet reached the HG-4 site. In the second survey, chromosomes with single FITC (HG-28TK) dots were identified and the status of the HG-4 probe was then determined. In this case those cells are detected in which HG-4 has already replicated but the replication fork has not yet reached the HG-28TK site. For both K562 and HL60, most of the molecules in this region replicate in the upstream direction, whereas in GM2568 replication appears to take place in the downstream direction. In all three cell types we detected many cells in which the single/double pattern was seen on all homologues, even in K562 which usually has three copies of chromosome 11. Examples of such nuclei from HL60 and K562 are shown in the photographs. It should be noted that in each experiment a small fraction of nuclei show an opposite hybridization pattern. This could represent an artefact due to inefficient hybridization of one of the probes, or could represent genuine cases in which the β -globin domain actually replicates in the opposite direction.

METHODS. Each cell line was grown to log phase and then subjected to centrifugal elutriation¹³. Nuclei from various fractions were hybridized *in situ* to the cos HG-4 (from E. Vanin) or cos HG-28TK (from F. Grosveld) probes. Fractions in which the globin domain was replicated in ~50% of the nuclei were then selected for double-label analysis. In the case of HL60 we used fraction 7, and for K562 fraction 4 from a previous elutriation experiment¹³. GM2568 was elutriated in a manner similar to HL60 and fractions 8 and 9 were subject to double-label replication analysis. The appearance of a double dot requires both DNA replication and sufficient segregation of the two sister chromatids in interphase nuclei. Our experiments therefore do not actually pinpoint the location of the replication fork, but rather the position at which the fork has proceeded far enough for the double hybridization signal of the replicated DNA to be distinguished in the wake of the replication machinery.

same probe will yield single hybridization dots if the locus has not yet undergone replication¹³. Using a double-label protocol, the replication state of both DNA at the β origin (cos HG-28TK) and that in the far 5' region (cos HG-4) on the same chromosome can be simultaneously visualized. In most cells, these two nearby probes should both appear either unreplicated or replicated, but in a few nuclei the replication fork will be located between the two probes and will reveal a single/double hybridization pattern on the same chromosome (Fig. 4). To amplify the number of these informative cells in the population, we used centrifugal elutriation to isolate cell fractions enriched for the appropriate stage in S phase in which the globin domain is undergoing active DNA synthesis. In 80% of these single/double nuclei (Fig. 4), it is the fragment closest to the β gene (HG-28TK) that replicates before the more distal probe (HG-4). These results, which are based on a completely independent assay, suggest that replication proceeds from the β gene outwards both in HL60 and K562, and are consistent with data obtained using the BrdU technique. We also assayed replication timing in the GM2568 cell line carrying the Hb Lepore deletion. In this case, replication proceeded in the opposite downstream direction, providing further proof that an alternative origin must be used in these cells.

In contrast to previous studies suggesting that the chicken histone H5 gene may be replicated from different origins in different cell types¹⁴, our findings show that replication of the human β -globin domain is initiated at a topographically fixed-origin locus, whose S-phase timing is under developmental control. In expressing cells this origin is fired early in the cycle, whereas in other cell types it replicates later^{4,5}. The precise timing of DNA synthesis initiation is probably regulated by a long-range *cis*-acting element which somehow interacts with this fixed origin, and there is some evidence that the upstream β -globin locus control region (LCR) sequence may have a role in this process¹⁵. In yeast, as well, origins must be under a similar type of control, because they can be made to replicate at different times in the cell cycle when transferred to other positions in the genome^{16,17}. This modular, di-elemental structure may represent a general model for the regulation of replication in eukaryotes. □

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A morphological biosensor for mammalian cells

Ivar Giaever and Charles R. Keese

An electrical biosensor is described that can continuously track morphological changes of adherent cells providing quantitative data from both sparse and confluent cultures. The method is capable of detecting vertical motion of cells of the order of 1 nm, much below the resolution of an optical microscope.

GREAT advances have been made over the past few years in quantifying biochemical and physiological activities in cultured cells. It has, however, been difficult to quantify changes of cell morphology, because most observations are performed with optical microscopes and are qualitative in nature. We have recently introduced a new method that we refer to as electrical cell-substrate impedance sensing, or ECIS for short, which can alleviate this problem. The system provides quantitative information about the morphology and motion of cultured cells¹⁻⁹. At the heart of the sensing device is a small gold electrode deposited on the bottom of tissue culture vessels and therefore immersed in ordinary tissue culture medium. When mammalian cells attach and spread on this electrode, its impedance changes because the cells restrict the current flow. The changes in impedance reveal important dynamical information about cell shape and movement. Since these properties are determined by the coordination of many cell functions, the measured impedance is extremely sensitive and responds to external conditions such as temperature, pH, and a myriad of biological and chemical compounds.

A schematic diagram of an ECIS system is shown in Fig. 1. In most measurements a 4,000-Hz signal with 1-V amplitude is applied to the sample through a 1 M Ω resistance creating an approximate constant current source. The lock-in amplifier can detect both magnitude and phase of the voltage appearing across the sample, providing additional information about cell morphology.

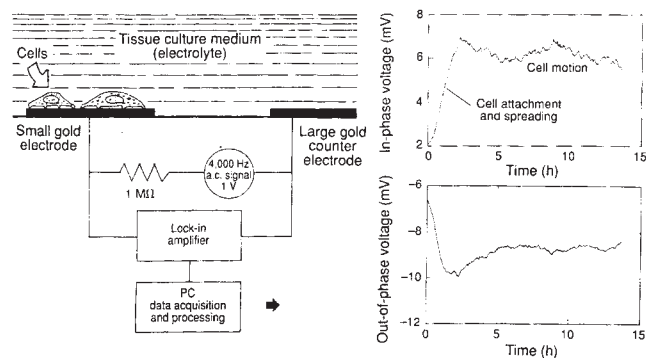


FIG. 1 A schematic of the ECIS instrumentation and a typical result. At time zero, human fibroblastic cells were inoculated in an ECIS well giving a final cell density of 10^5 per cm^2 . Both the in- and out-of-phase voltages are shown as a function of time.

An IBM-compatible personal computer is used both to run the experiments and to record and process the data. The method can provide a continuous stream of data and is therefore suited to automation of many of the common observations carried out in tissue culture.

Data obtained from a typical cell inoculation are also shown in Fig. 1. Both the in-phase and out-of-phase voltage change when the cells attach and spread on the small electrode. The physical explanation is that the cells act as insulating particles because of their plasma membrane and interfere with the space immediately above the electrode available for current flow. It is important to note that the cells play a passive role by blocking the current, and tiny plastic chips placed on the electrode cause similar increases in the impedance. After the cells are fully spread the measured impedance continues to fluctuate. These fluctuations are the result of the constant motion of the cells altering the current flow in subtle ways. Both the final average impedance values and the fluctuations are characteristic for the cell type and may be used as a cell signature or fingerprint.

Cell attachment

The ECIS system has several direct applications useful for animal cell culture. Figure 2 shows an example of an attachment/spreading assay. Here, each electrode has been precoated with an adsorbed pure protein layer before exposing it to the tissue culture

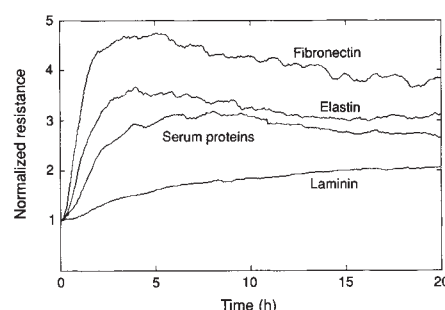


FIG. 2 Proteins were adsorbed on four ECIS electrodes, and a cell suspension of Dunning prostatic adenocarcinoma cells (subline G; 10^5 cells per cm^2) were added at time zero. The in- and out-of-phase voltages were converted to resistance and capacitance treating the system as a series RC circuit. The resistance normalized to the value measured at time zero is plotted.

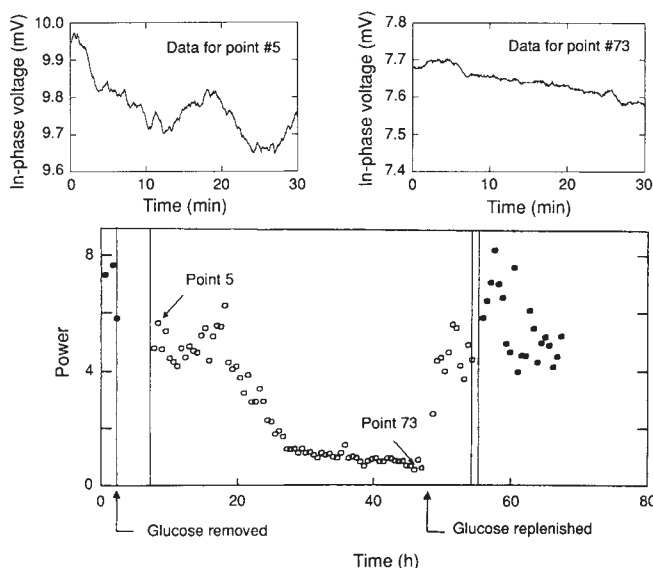
one set of cells, and the process is completely automated⁴.

Cell motion

The ECIS method readily detects changes in impedance in sparse cultures as cells move on or off the small electrodes. More interesting is the fact that similar fluctuations continue after the cells have reached final confluence and are contact inhibited. The fluctuations are now due to vertical motions of the cells and changes in the transcellular resistance of the layer. Model calculations show that an average change in the distance between the ventral surface of the cells and the substrate of less than 1 nm is easily detected, much better than the resolution of an optical microscope⁵. It is both this sensitivity and the fact that vertical motion is detected that make this a unique measurement of motility.

Pictured in Fig. 3 are results of an experiment demonstrating that the observed motion in a confluent layer is directly related to cell metabolism. Two hours into the experiment all glucose and other reduced carbon sources are removed from the medium for an extended time. Each point in the figure is a measure of the power (variance) of the in-phase voltage fluctuations measured over a period of about 30 min. For example, the two smaller graphs show the actual voltage fluctuations used to calculate the power reported by the points indicated in the main graph. The

FIG. 3 Analysis of fluctuations obtained from a confluent layer of WI-38/VA13 cells as energy supplies in the medium are manipulated. The vertical lines encompass regions of the curve where no data were taken. The points (filled circles) before and after the first and last vertical line, respectively, are taken of cells in complete medium with fetal serum. The other points (open circles) are of cells in a balanced salt solution with and without glucose as shown (see text for more details).



voltage fluctuations diminish when the cells run out of stored energy reserves (roughly 18 h after glucose deprivation) but regain their vigour when nutrients are replenished in the medium. The results suggest that the variance of the fluctuations may be taken as an indication of the general cell health and can be used to follow the cells in real time⁸.

Effects of chemical compounds

ECIS, in addition to monitoring cell behaviour, can be used to quantify effects of chemical compounds and has the potential as an alternative to animal testing for toxicology studies⁶. Because changes in cell morphology or motion clearly depend upon many different chemical reactions taking place in the cells, a variety of compounds cause dramatic cell responses. Figure 4 illustrates some of the observed effects of three different compounds on three different confluent cell layers.

Figure 4a shows a time course of the effect of the addition of thrombin upon endothelial cells⁷. The observed resistance decrease can have two distinct causes — the cells may have moved further away from the substrate or the transcellular resistance has decreased. By simultaneously measuring in- and out-of-phase voltages, these two effects

can be untangled using a model calculation. In this case, the thrombin-induced effect results mainly from a decrease in the transcellular resistance of the cell layer.

Figure 4b shows the response of murine macrophages to heat-killed *Listeria monocytogenes*⁵. The rise in resistance is due to activation of macrophages and illustrates that their morphology has changed to a more flattened shape. Finally, after several hours, the cells will die as a result of a very acidified medium.

The final example in Fig. 4c illustrates how epithelial cells (MDCK) respond to Triton X-100. There is a clear reproducible change in resistance corresponding to different levels of the detergent. It is interesting that these cells show a large excitatory response with increased impedance fluctuation activity at intermediate concentrations of detergent (in this case at 40 $\mu\text{g ml}^{-1}$).

Discussion

Tissue culture is a valuable and rich scientific endeavour, but as presently practised most morphological measurements are time consuming and rather descriptive. Electrical cell-substrate impedance sensing, described in this short review, has the potential to improve this. It appears to us that ECIS can open up a new avenue in the field of tissue culture.

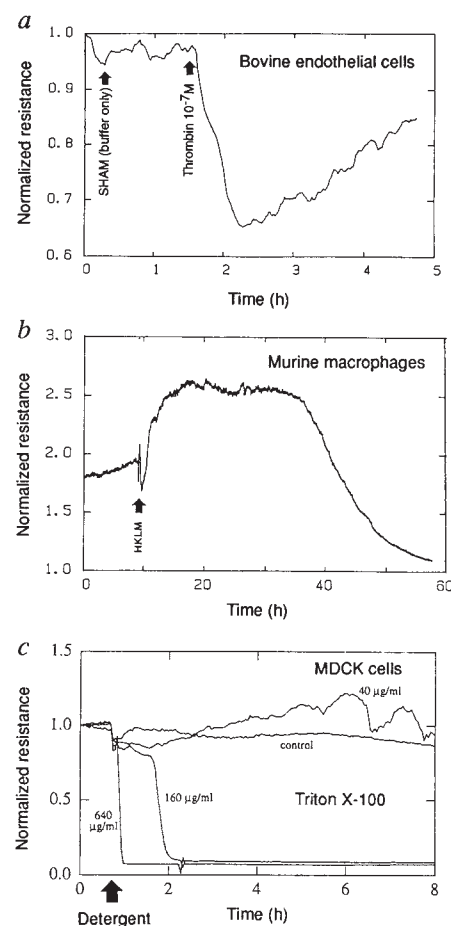


FIG. 4 See text for details. Resistance values calculated as in Fig. 2 are normalized to values at the start of the data shown for Figs 4a and 4c and to the value of the cell-free electrode in Fig. 4b.

Unlike most conventional morphology measurements of cells in tissue culture, these results are naturally quantitative, these data are taken in real time and in a continuous fashion, and all measurements are computer-controlled. While we envision many potential applications and are presently exploring several in our laboratory, we recognize that in the long run the final judgment regarding the efficacy of this method must come from experts who routinely use tissue culture in biological and biomedical applications. □

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Ivar Giaever is in the School of Science and Charles R. Keese is in the Department of Biology at Rensselaer Polytechnic Institute, Troy, New York 12180-3590, USA. In 1991, I. G. and C. R. K. founded Applied BioPhysics, Inc., 1223 Peoples Avenue, Troy, New York 12180, USA to commercialize and explore applications of ECIS. I. G. and C. R. K. are supported in these endeavours by SBIR grants from the National Institutes of Health. For more information on the ECIS system, fill in reader service number 100.

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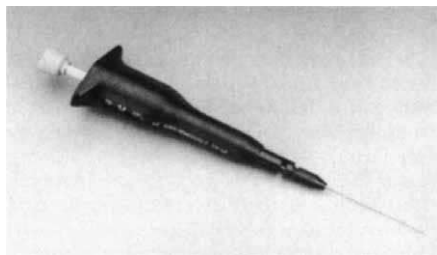
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News you can use

New products for the cell biologist worth noting this week include a nonradioactive method for detecting cell culture contamination, a medium for the production and maintenance of B-cell hybridomas and a high-pressure homogenizer.

DRUMMOND Scientific's new **manual oocyte microinjection pipette** for the deposition of DNA is said to be as steady as a rock by virtue of the nonrotating plunger (*Reader*

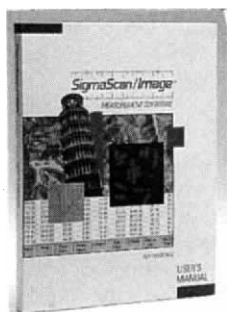


Drummond's microinjection device.

Service No. 101). Volumes of 30 nl or more can be injected. The device utilizes pulled capillary tips and is supplied with 100 8-inch glass capillaries — said to be sufficient for preparing 200 tips. Replacement parts are available, including 3.5- and 8-inch glass 30G/2-inch needles.

Enzyme-linked immunosorbent **assays for the diagnosis of Hantavirus infections** are now available from Progen Biotechnik (*Reader Service No. 102*). The Hantavirus- μ -capture-ELISA uses recombinant nucleocapsid antigens and monoclonal antibodies specific for Hantaan and Puumala subtypes. Progen says that the reaction pattern obtained with the two antigens allows the identification of the infecting virus type as Puumala or Hantaan-like and should, therefore, have prognostic value. The Hantaan and Puumala IgG ELISA tests are solid-phase assays based on recombinant nucleocapsid antigen-coated microstrips.

Jandel Scientific says that you can throw out your rulers, protractors and planimeters since it is now shipping **SigmaScan/IMAGE measurement software** for Windows 3.1 (*Reader Service No. 103*). With this package, users can make a variety of morphology and intensity measurements directly from images displayed on their PC monitor using

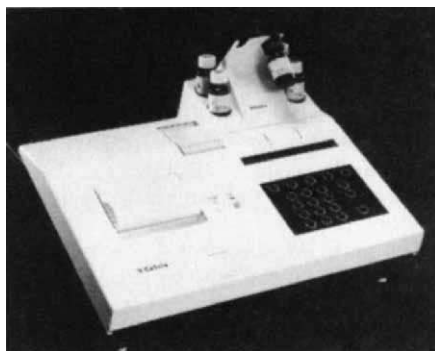


Measurement tools.

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monochrome image files can be loaded from scanners, disk or CD-ROM, which is likely to be convenient when working with X rays, electrophoresis gels, photographs and images saved in digital formats directly from instruments. SigmaScan/IMAGE supports TIFF, BMP, TGA, PCX and GIF image file formats and runs on IBM PCs and compatible computers. The system's measurement features include perimeter, area, distance, angle, slope, x-y coordinates, compactness, feret diameter, shape factor, major/minor axes (length, slope, endpoint coordinates), centre of mass, running tally, width-averaged line intensity, area-averaged intensity, and point intensity. The US\$495 package requires 4 MB RAM, 3 MB free disk space plus room for images, a 256-colour VGA monitor, and a Microsoft-compatible mouse. A maths coprocessor is recommended.

As a complement to its new range of bioluminescence reagents, Celsis, in conjunction with MGM Instruments, has customized the Optocomp I **photon-counting luminometer** so that it makes economical use of reagents while cutting down on cocktail waste (*Reader Service No. 104*). The



Optocomp I luminometer — see Celsis.

photomultiplier tube detector has, says Celsis, been selected for its stability, sensitivity and wide dynamic range. Photon events generated by the bioluminescence reaction are detected by the photomultiplier, recorded by high-speed digital circuits and then stored in memory for further processing. Up to 30 protocols can be stored in memory: the front panel displays information and prompts the user through all stages of operation and programming. Results are reported in both counts and concentration levels.

■ Cell analysis

The **monoclonal antibody reagent for cell analysis**, Anti-IL-2R p75, the first in a series of new research reagents currently being developed by Becton Dickinson Immunocytometry Systems, is designed for pheno-



Anti-IL-2R p75 available as purified antibody or phycoerythrin conjugate.

typic and functional studies of natural killer (NK) lymphocytes, as well as studies of ligand-receptor interaction, IL-2 receptor expression and function, and lymphocyte activation (*Reader Service No. 105*). Anti-IL-2R p75 is supplied as a purified antibody and phycoerythrin conjugate that is ready-to-use in research experiments that incorporate flow cytometry and other cell analysis techniques. It inhibits the binding of IL-2 to the IL-2 receptor and is said not to react with the low affinity IL-2R p55 subunit (CD25 or Tac antigen). Instead, the reagents are specific for the 75-kD subunit for the high affinity IL-2 receptor, which transduces growth signals for T lymphocytes, NK lymphocytes and monocytes. Moreover, the company says that in its purified form, Anti-IL-2R p75 contains a special formulation with the concentration of sodium azide reduced to 0.01 per cent (this minute concentration of azide is designed to minimize inhibitory effects in functional experiments). In addition, the absence of carrier protein allows Anti-IL-2R p75 to be used in affinity chromatography, biotinylation, conjugation, immunoassays and immunoprecipitation.

Measuring just one square foot, the new **Z1 particle counter** from Coulter can be used to count anything from algae and bacteria to platelets, spermatozoa and yeasts

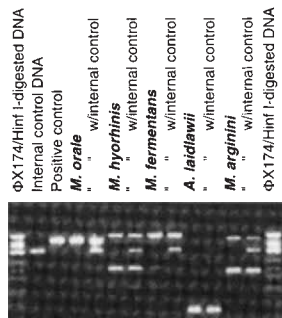


The Z1 counts particles from 1 to 120 μ m.

with a size range ranging from 1 μm to 120 μm (*Reader Service No. 106*). Various optional aperture tubes are offered including 50 μm , 100 μm , 140 μm and 200 μm . Thresholds are entered by a small keypad and results are displayed on an integral LCD which leads the user through the programme step by step. Results can also be output through an optional printer, which can present the analysis as a complete per-run report or as a line-by-line presentation that is formatted for a simplified analysis comparison. Coulter says that the Z1 has been designed to eliminate calibration drift and will determine particle counts above the lower threshold, above the upper threshold and between thresholds. Metered volumes include 100 μl , 500 μl and 1,000 μl . The Z1 also incorporates an oil displacement pump that allows for the elimination of mercury from the system and has a stated accuracy in the ± 1 per cent range.

■ Coping with contamination

With the Mycoplasma PCR Primer Set, Stratagene says that it has developed a **nonradioactive method for detecting Mycoplasma infection** in tissue culture cells in about 4 h (*Reader Service No. 107*). With this kit, there is no need to carry or maintain a special indicator cell line, use radioactivity or a fluorescent microscope. Included with the PCR primers are both positive control and competitive internal control templates: the positive control is noninfectious, genomic mycoplasma DNA which is used for comparison of the test results; the internal control confirms polymerase activity. The Mycoplasma Primer Set is supplied with enough PCR primers, internal control template and StrataClean resin for 50 determinations and positive control template for 10 determinations.



Mycoplasma PCR products gel.

Wak-Chemie's new Biocidal ZF **spray disinfectant** for cleaning surfaces and incubators in cell culture laboratories is biodegradable and free of aldehydes (*Reader Service No. 108*). Biocidal ZF is said to be effective against fungi, spore-forming bacteria (also spore-forming organisms) and enveloped viruses, and is intended for the disinfection of incubators and laminar flow hoods, laboratory instruments and the outside surfaces of tissue culture bottles.

■ Happy medium

Neurobasal medium from Life Technologies supports the **long-term growth of neurons and neuroblastomas** (*Reader Service*

No. 109). When combined with the company's B-27 Supplement and L-glutamine, the company says that a 60–90 per cent viability of rat embryonic hippocampal neurons has been achieved — even after 4 weeks in culture. In addition, the growth of glial cells was said to be reduced to less than 0.5 per cent. Life Technologies says that the Neurobasal/B-27 combination has also been shown to support the growth of neurons from embryonic rat striatum, substantia nigra, septum, cortex and neonatal dentate gyrus and cerebellum. This combination also supports the growth of many neuroblastomas.

PromoCell is offering cell culture systems with **human primary cells and cell-specific serum-free medium** (*Reader Service No. 110*). Each of the company's serum-free media has been specially formulated to meet the requirements of specific types of

Complete cell culture systems with human primary cells and cell-specific serum-free media are now being offered by German-based PromoCell.



cells. It has been supplemented with growth factors, tested for growth and cloning efficiency using primary cell cultures isolated at PromoCell, produced according to GMP guidelines, and is supplied ready for use. In addition to products like EpiKit, EndoKit, KGM or EGM, the 1993–1994 catalogue also lists a range of new products, such as BEGM, a serum-free medium for bronchial epithelial cells, SmGM for smooth muscle cells and SkGM for skeletal muscle cells.

When proteolytic degradation is suspected in your diagnostic or cell culture applications, Miles says that it may have the answer with its Trasylol VLE **serine protease inhibitor** (*Reader Service No. 111*). Trasylol VLE is said to be active against most biologically significant proteases, stable under extreme environmental conditions, compatible with enzyme and cellular systems and effective at very low dose levels.

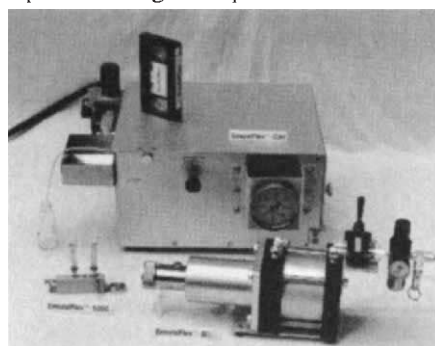
New from the Amimed-line of BioConcept — IP301 and SF-1 **baculovirus insect media** (*Reader Service No. 112*). The media were developed for the serum-free and/or low protein cultivation of insect cells. Both can be used for the culture of Sf9 as well as Sf21 insect cell lines and will find application in the production of recombinant proteins in the baculovirus expression system.

IP301 is a chemically defined and optimally balanced nutritional environment, supplemented with defined lipids and Pluronic F68. SF-1 medium is based on IP301 but is supplemented with ultrafiltered protein hydrolysates. IP301 medium is optimized for the growth of insect cells in both small cultures as well as batch cultures, whereas SF-1 is said to be most useful for larger spinner and batch cultures as well as scale-up perfusion fermentation processes.

Hybridoma cloning factor — a medium rich in growth factors which can be used for the **preparation, production and maintenance of B-cell hybridomas** — is available from Metachem Diagnostics (*Reader Service No. 113*). Use of hybridoma cloning factor medium is designed to eliminate the need for feeder cell layers and re-feeding during cloning, as well as the need for individual growth factors or growth supplements. Use of hybridoma cloning factor is said to increase the number of hybridomas that survive HAT (hypoxanthine, aminopterin, thymidine) selection, increase the number of hybridomas that secrete antibody and enhance the cloning efficiency of hybridomas.

■ Cell manipulation devices

With prices starting at under US\$4,000, the new EmulsiFlex **high-pressure homogenizer and cell disrupter** from Avestin should find use in the production of emulsions, liposomes and for cell rupture (*Reader Service No. 114*). Pressure can be adjusted from zero to 30,000 p.s.i. The instrument has a capacity that can range from as low as 1 ml up to several gallons per hour. The device



Avestin's high-pressure homogenizer.

has a dynamic valve structure allowing scale up with constant pressure by different flow rates, and a stainless steel heat exchanger. Optional extras include automatic temperature control and computer-controlled pressure adjustment.

The ECM 200 **cell fusion system** from BTX Electronic Genetics combines two systems — electrofusion and electroporation — in one instrument (*Reader Service No. 115*). The device, which operates in either manual or automatic mode, includes a 1 MHz a.c. wave form for rapid cell alignment and a high-voltage square-wave pulse

for electrofusion and electroporation. The system also includes a graphic pulse analyser, called the Optimizer, which monitors the chamber conditions during electroporation and electrofusion. Included with the system are microslides that have parallel electrodes adhered to glass to enable direct viewing under a microscope. It is also available as an embryo manipulation system.

The two new **electropulsators** from Jouan, the PS10 and PS15, offer square-wave output with constant electric field, a variable voltage and number of pulses without protracted recharging times, and mobile parallel electrodes that are resistant to corrosion (*Reader Service No. 116*). The devices can be used for a variety of applications



Jouan's electropulsator — for fusion, transformation and insertion applications.

(fusion, transformation, insertion, efflux of metabolites) and with various cell types including yeasts and whole bacteria, and whatever the configuration (plated, suspension, microcarrier or continuous flow). The stated transformation rate is up to 60 per cent.

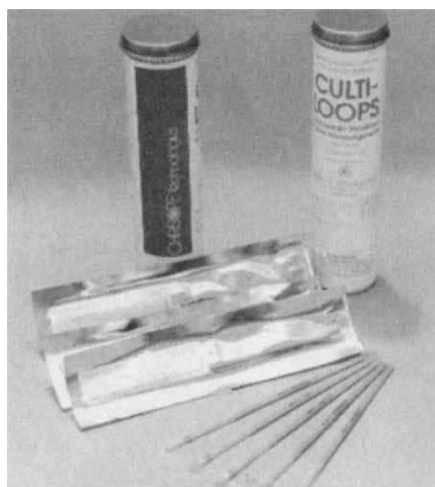
■ Assorted tools of the trade

Cellon's new Cytolift **airlift bioreactor** for cell and tissue culture is suitable for suspension culture, microcarrier culture of anchorage-dependent cells and photosynthetic culture of plant cells and blue-green algae (*Reader Service No. 117*). The 580-ml bioreactor column is fitted with a glass water jacket as standard to enable culture at elevated or subambient temperatures.

The new Bio-Color **automated slide staining machine** should, says Fred Baker, allow users to standardize staining procedures and make cost savings in terms of reagents (*Reader Service No. 118*). The instrument can store up to 30 different staining cycles with users programming the times for staining and rinsing, with or without agitation and drying. Staining times can be specified in seconds and/or minutes. The system has three preprogrammed cycles for 'Grams' with the option for May-Grunwald-Giemsa type stains and other 'special' cycles. Rinse water is kept flowing constantly through the chamber in which the stain baths are seated; this, the company says, ensures that washing between staining cycles is thorough, with fresh water used every time. Eight staining baths are available and twenty slides can be processed per cycle.

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Unipath recently launched a range of ready-to-use, **disposable bacteriological loops** that contain viable micro-organisms (derived from original American Type Culture Collection stock cultures) held in a gelatin-based film within the loop (*Reader Service No. 119*). The film has been dried to

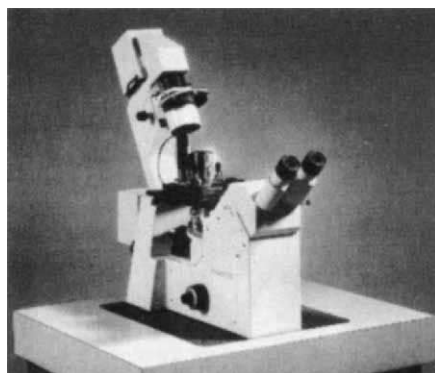


Ready-to-inoculate micro-organisms for quality-control testing.

maintain the viability of the micro-organisms and rehydrates on contact with both warmth and moisture. Stated uses of the Culti-Loops include performance testing of culture media, stains, diagnostic kits and reagents, the evaluation of bacteriological procedures and the maintenance of stock cultures. The loops are designed for quick and easy use: nonfastidious organisms can be streaked directly onto the culture medium and will inoculate as many as five plates; for fastidious organisms, Unipath says that it is best to resuspend the film in liquid medium before streaking. Each plastic loop is individually foil-wrapped and packaged in cans of 10.

■ Small-world solutions

The new TopoMetrix Explorer Life Sciences **atomic force microscope** (AFM), which combines the AFM with the high-resolution inverted optical microscope, should find application in cell biology, biophysics and molecular biology (*Reader Service No. 120*). The Explorer integrates high-magnification imaging (up to $\times 10^9$)



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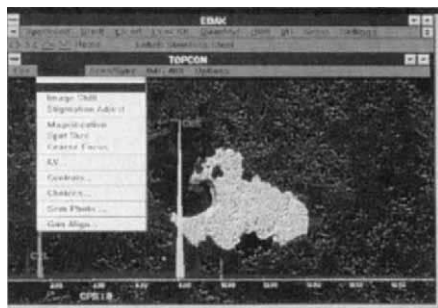
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Reader Service No.12

PRODUCT REVIEW

and three-dimensional, quantitative measurement capabilities of an AFM with any one of several commercial inverted optical microscopes, including Zeiss, Nikon and Olympus models. The high magnification of the inverted microscope is designed to complement the imaging capabilities of AFM by providing independent monitoring of tip-sample interaction. It is also used in positioning the AFM tip and sample. The system incorporates a new scanner design, which operates in liquid without the use of separate fluid cell hardware; this, TopoMetrix says, eliminates meniscus forces and allows imaging to be done at much lower forces than when performed in ambient air. The One-Pass Imaging capability allows simultaneous acquisition of up to eight channels of data in a single scan. New preparation techniques enable samples to be firmly anchored while being scanned under liquids. These technologies, combined with the liquid scanning techniques, allow the Explorer to be used on living systems under physiological conditions. Lateral force, modulated force and noncontact traditional imaging modes are offered.

TopCon's new integrated **scanning electron microscope/energy dispersive (X-ray) spectrometer (SEM/EDS)**, which incorporates EDAX EDS, is operated using one computer, one mouse and one keyboard.



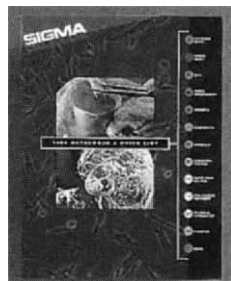
An EDAX EDS spectrum overlaid on an SEM image — see TopCon.

(Reader Service No. 121). An EDS adds the ability of performing elemental analysis on an SEM's high-resolution images of surfaces. With this system, the SEM image and

EDS spectrum appear on a single screen and are controlled by one mouse and one keyboard. The EDS spectrum can be overlaid on a full-screen SEM image or appear side-by-side with the SEM image. Multiple windows can be brought up in any combination (SEM menus, EDS menus, SEM images, EDS spectra), and menus and windows can be moved or hidden. With TopCon's Dual Control feature, the user can simultaneously control the SEM from the operating panel and the EDS software from the computer, eliminating the time wasted switching between SEM and EDS operation software. Because only the EDAX EDS computer is used in the integrated SEM/EDS system, TopCon says that there is no duplication of control systems. When the SEM/EDS is not in use, the computer can still be used for word processing.

Catalogues

Copies of Sigma's 1994 **catalogue for cell culture** are now being distributed and feature over 150 new products (Reader Service No. 122). New product listings in the mammalian cell culture line include large-volume media bags for use with any of the company's standard media or custom-prepared formulations, iron-supplemented calf serum, Histopaque in 500-ml bottles, a *Mycoplasma* detection kit, β -interferon and leukaemia inhibitory factor. Plant growth regulator kits and liquid plaquing insect media are new to the plant and insect cell culture lines.



What's new for 1994?

The latest BioGenex catalogue, which contains a host of **cell pathology reagents and products** designed to provide consistent and reproducible immunohistochemical staining, can now be obtained through Biomen, the UK division of Menarini Diagnostics (Reader Service No. 123). New product listings include prognostic and proliferation markers, an expanded range of CD

markers, and an *in situ* hybridization kit for the detection of kappa and lambda mRNA in fixed-tissue sections.

Kits

Process up to 12 samples in 20 min, says J. T. Baker, with its ScreenMax mini-prep kit for the purification of plasmid samples (Reader Service No. 124). In just three main steps — boiling, precipitation and washing



J.T. Baker's mini-prep kit designed to simplify purification; speed throughput.

— the kit is said to provide clean extract yields of 2 μ g to 7.5 μ g from only 0.2 ml of culture. No hazardous chemicals are necessary; only a heating block and mini-centrifuge are required. The kit has sufficient reagents for 100 mini-preps.

With the lysate ribonuclease protection kit for the detection and **characterization of RNA from guanidine thiocyanate cell lysates**, now available from United States Biochemical, the entire procedure can be carried out in a single reaction tube (Reader Service No. 125). USB says that purification of RNA is not required with this method as guanidine thiocyanate inhibits RNases and promotes molecular hybridization. This then eliminates the uncertainty associated with RNA integrity and yield during RNA purification. Additionally, because hybridization is accomplished with excess probe in solution, this method can be used for quantitative analysis. The US\$225 kit includes sufficient reagents for 100 assays. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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